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# The Effects of *p*-Aminobenzoic Acid, Pantothenic Acid and Pyridoxin upon Respiration of *Neurospora*<sup>1</sup>

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## INTRODUCTION

It is known that *p*-aminobenzoic acid, pantothenic acid and pyridoxin belong to the B-complex vitamins which are growth-factors for animals, plants and some micro-organisms. Specific respiratory functions of several B-vitamins are known. Thiamin pyrophosphate is the prosthetic group of carboxylase and possibly of pyruvic acid dehydrogenase. B<sub>2</sub> is present in the prosthetic group of the alloxazine enzymes, nicotinic acid is part of the pyridine-nucleotides, including cozymase. It has also been shown that addition of certain vitamins known to be components of respiratory enzymes to organisms insufficiently supplied with these may strikingly accelerate respiration. Pantothenic acid may act in a similar way for yeast (Williams, Mosher, and Rohrman, 1936), and for *Proteus morganii* (Dorfman, Burkman, and Koser, 1936; Hills, 1943).

An opportunity to test the possibility that certain vitamins of the B-group may be similarly related to respiratory enzymes presented itself when mutant strains of *Neurospora* requiring *p*-aminobenzoic acid, pantothenic acid and pyridoxine were obtained (Beadle and Tatum, 1941; Tatum and Beadle, 1942a, 1942b).

Wild-type *N. crassa* and *N. sitophila* need only biotin in addition to sucrose, ammonium nitrogen and inorganic salts for growth. Each of the mutant strains requires in addition the particular vitamin which it can no longer synthesize. The *thiaminless* mutant requires B<sub>1</sub>; *pyridoxineless*, B<sub>6</sub>, etc. Excepting for their vitamin requirement each mutant

<sup>1</sup> This research was supported in part by funds provided by the Rockefeller Foundation. The authors are indebted to Dr. G. W. Beadle and other members of the genetics group for making available mutant strains of *Neurospora*. To Miss Margaret Briggs and Mr. Francis Haxo they are indebted for help with some of the respiratory determinations, and to Mr. James Horst for some of the alcohol and fat determinations.

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strain is essentially normal and, when supplied with the required vitamin, its growth is a function of the vitamin concentration. With a completely adequate vitamin supply growth occurs at the same rate and to the same extent for a given carbon-source as in the wild type. An excess of vitamin will not increase the rate of growth of the mutant, nor will the addition of vitamins increase that of wild type (Beadle and Tatum, 1941).

### EXPERIMENTAL

*Materials and Methods.* To determine whether a vitamin has a probable respiratory function, it is necessary to demonstrate a change in the rate of respiration following its addition to the appropriate mutant in a state of vitamin deficiency. Standard Barcroft-Warburg techniques were used in following respiratory rates in this approach.

The following mutant strains of *Neurospora* were used: *N. sitophila* 299 *pyridoxineless* (Beadle and Tatum, 1941; Stokes, Foster and Woodward, 1943); *N. crassa* 1633 *p-aminobenzoicless* (Tatum and Beadle, 1942a); and *N. crassa* 5531 *pantothenicless* (Tatum and Beadle, 1942b). The cultures were grown at 25° C. in 125 ml. flasks containing 50 ml. medium (Horowitz and Beadle, 1943) inoculated with fresh conidiospores. It was found necessary to maintain a given ratio of nutrient medium to flask volume and to shake the cultures continuously during incubation to obtain reasonably comparable and uniform growth. The  $Q_{O_2}$  of cultures grown in this manner varied from 10–55 mm.<sup>3</sup> O<sub>2</sub> per hr. per mg. dry wt. on different days. The age of the culture was apparently the most important variable for, in a series of cultures inoculated at the same time, very young cultures gave high values, the value declining with age. It was not possible to predict by the period of incubation the “physiological age” of the culture.

Under a variety of experimental conditions tried, it proved impossible to grow *Neurospora* in the “yeast-like” form as in the case of other fungi (Kluyver and Perquin, 1933). Fragmentation of mycelium with a butter homogenizer was also tried, but the respiration was reduced to only a few per cent of the control.<sup>3</sup> All subsequent experiments were therefore performed with clumps of mycelium, the dry weights of which were later determined.

Unless otherwise indicated the mycelium was always suspended in medium containing sucrose and all the salts which *Neurospora* requires for growth.<sup>4</sup> The rate of respiration was relatively constant over a period of several hours in such a medium indicating that during this period growth was insufficient to interfere with the measurements of respiration. (Note the straight lines for rate of respiration in Fig. 1.)

Mycelium obtained from a single 24-hour culture at times showed almost no variation but sometimes showed as much as 60% variation in respiration of different samples. With older cultures the variation was even greater. It thus became necessary to

<sup>3</sup> Even crushing the mycelium between the fingers in some cases reduced the rate of respiration by 75%.

<sup>4</sup> Mycelium in distilled water or in buffered balanced salt medium respired at the same rate as in the medium containing ammonium salt needed for growth.

conduct experiments in such a way that a control could be established in each Warburg vessel before additions of substrate or vitamin so that, in spite of the individual variations, the percentage change produced by additions could be significantly determined.

*Experiments Comparing the Rate of Respiration of Wild Type and Mutant Strains.* Before studying the effect of vitamins upon respiration of the mutant strains, it seemed desirable to determine whether any differences were to be observed between the respiration of the mutants and the wild type. The results are summarized in Table I. It is apparent

TABLE 1

*Comparison of Respiration of Wild-Type and Mutant Strains of Neurospora  $QO_2$  in mm.<sup>3</sup> per Hour per mg. Dry Weight*

Strain	Average $QO_2$	Range $QO_2$	Strain morphological mutants	Average $QO_2$
Wild type	32.0	15.1-56.0	2499	16.0
<i>p</i> -Aminobenzoicless (1633)	21.3	11.7-31.2	5827	19.6
Pyridoxineless (299)	27.4	18.7-35.6	5452	19.6
Pantothenicless (5531)	19.5	15.1-26.8	5801	29.4
Thiaminless (1090)	26.6	16.0-35.6	3100	15.3
Nicotinicless (4540)	32.8	25.0-41.0	30210	22.3

that the respiration of the vitamin mutants and wild type is of the same order of magnitude. This is not surprising in view of the fact that rate of growth of these mutants when supplied with the needed vitamin is comparable to that of the wild type.

It seemed interesting to study the rate of respiration of some of the "stunted" morphological mutants in this connection, since their rate of growth is much slower than that of the wild type. The results in the right-hand column on Table I indicate that the rate of respiration is of the same order of magnitude as that of the wild type, although the average for the group of mutant forms is somewhat lower. Apparently the morphological mutants are unable to use the nutrients or their products for growth as efficiently as can the wild type or the biochemical mutant strains.

When vitamins are added to a wild type culture, there is no change in respiration (Fig. 1). Experiments were tried adding  $B_1$ , *p*-aminobenzoic acid, pantothenic acid,  $B_6$  and a mixture of all the vitamins of the B-complex, including some factors not yet fully established in the recognized complex. Cultures grown in inadequate sugar respond to its

addition, but there is no further increase when a vitamin or a mixture of vitamins is added. This indicates that in healthy wild type cultures, the rate of respiration is not limited by the concentration of vitamins of the B-complex. In other words, the cell supplies all it can use. Some of the data are shown in Fig. 2. Similarly, there is no accelerating effect of addition of vitamins to mutant strains grown in an adequate supply of vitamins and sucrose as shown in Fig. 1.

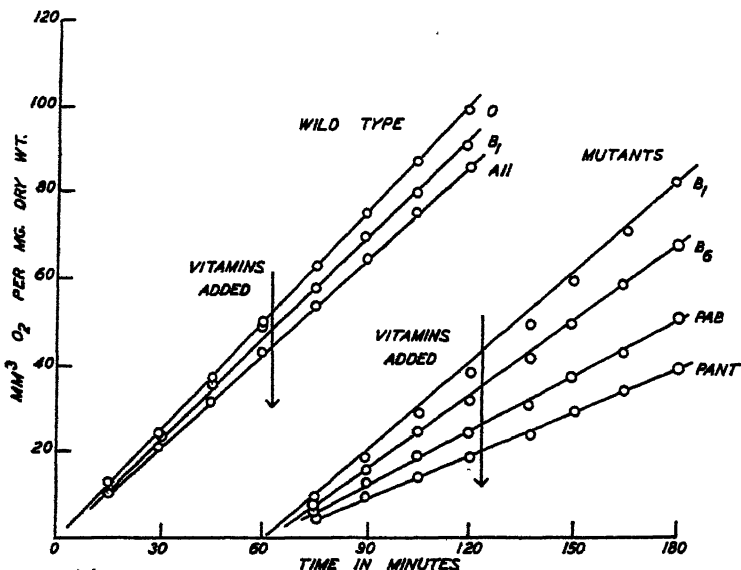


FIG. 1

Effect on Respiratory Rate of Addition of Vitamins to Wild Type and to Mutant Cultures Grown in Adequate Nutrient and Vitamin Medium

*Experiments on Vitamin Deficient Cultures.* The accelerating effect on respiration of vitamin B<sub>1</sub> is well known for yeast and brain tissue. A few studies were therefore made adding vitamin B<sub>1</sub> to the thiaminless mutant of *Neurospora*. Such an addition resulted in a small but significant change in slope, indicating an increase in the rate of respiration. The results are summarized in Fig. 3 and Table II. An average increase of about 40% was obtained in the experiments tried. The increase in rate occurred fairly soon after the addition of the vitamin.

When *p*-aminobenzoic acid was added to a culture of the correspond-

ing mutant starved for this vitamin, an increase in the rate of respiration was observed, the average being about 27.5%. Similar experiments were performed with pantothenic acid and pyridoxin with essentially similar results; the average increase in the first case was 25%, in the second 36%. The data are summarized in Figs. 3, 4 and 5 and Table II. If the vitamin mutant strain is starved for sucrose but not for vitamin,

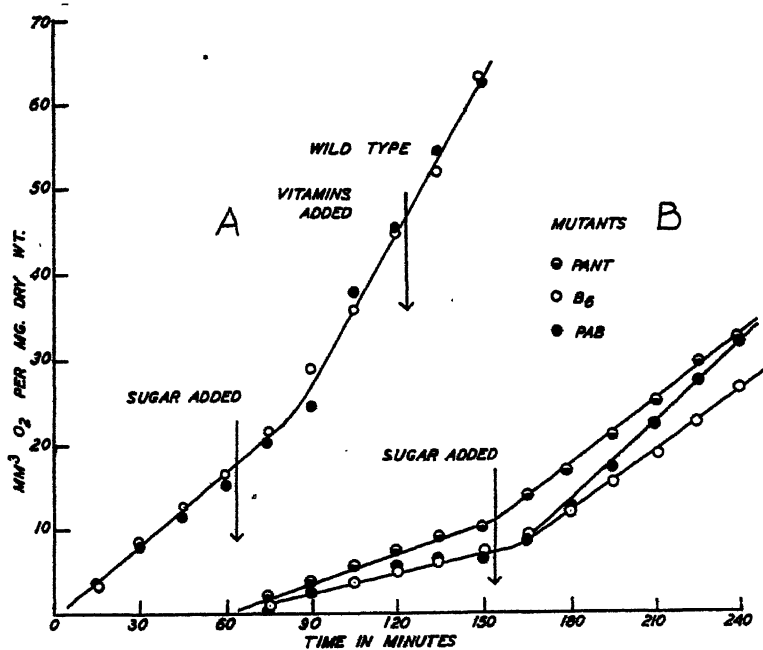


FIG. 2

Effect on Respiratory Rate of Addition of Sucrose at "A" \* to Wild Type and at "B" to Mutant Cultures Starved by Growth on Inadequate Sucrose

there is an acceleration of respiration after adding sucrose (Fig. 2). However, if the mutant strain is starved for both sucrose and vitamin, the rate of respiration increases following the addition of both vitamin and sucrose (see Fig. 2). If the cultures are starved too long for either vitamin or sucrose or especially for both, the mycelium tends to disintegrate and there is but little response to any additions. The additions are then followed by a slow gradual upswing in the respiration which is

\* In "A" vitamins were added only to the culture shown by solid black circles.

not pronounced for several hours. Unfortunately, it was difficult, even under what seemed to be the same conditions, to consistently obtain a satisfactory degree of starvation for both carbohydrate and vitamin.

From these results it is apparent that at least two factors limit the rate of respiration: (a) the amount of available substrate, and (b) the amounts of the limiting vitamin supplied. The observed increase in respiratory rate following the additions of vitamin might be due to (1) use of the vitamin as a nutrient; (2) growth of the mycelium as a whole;

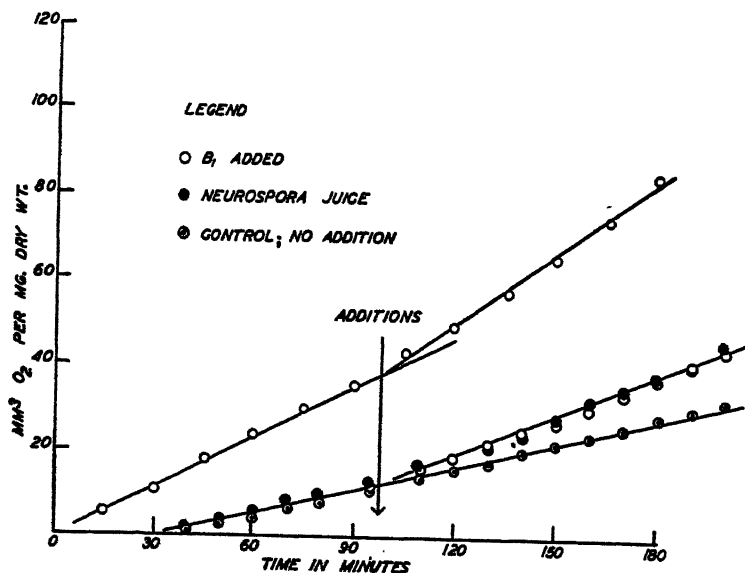


FIG. 3

Effect on Respiratory Rate of the Addition of Vitamin  $B_1$  to the Thiaminless Mutant Starved by Growth in Inadequate Vitamin.

(3) direct involvement of these vitamins in enzymes catalyzing reactions resulting in oxygen consumption; or (4) indirect effects resulting in greater concentrations or activities of the enzymes concerned in oxygen uptake. As such minute quantities of vitamins are used and the effect is so lasting, the first possibility is excluded. The second possibility seems unlikely, for the increase in respiration occurs in from 15-45 minutes and an increase in mycelium corresponding to a 25-40% increase in rate of respiration is known to be impossible during this time.

However, if the effect of a vitamin involves a specific increase in some particular enzyme system, the response to the vitamin addition might be expected to be fairly rapid. The response to the addition of B<sub>1</sub> was almost immediate; in the case of the other three vitamins it was somewhat slower, requiring 15–45 minutes. This slower response was not due to an inadequate supply of the vitamin as concentrations in excess of the minimal amounts did not speed up the response. Thus, in an experiment with the *p*-aminobenzoicless mutant 20, 2 and 0.2  $\gamma$  of *p*-aminobenzoic acid caused a simultaneous and equal increase of 56% in the rate of respiration, while 0.002  $\gamma$  produced no effect. Similar results

TABLE 2

*Effect on the Rate of Respiration of Addition of Vitamins to Mutant Strains of Neurospora Grown on Inadequate Amounts of Vitamin*

Mutant strain	Av. Qo <sub>2</sub> starved	Av. Qo <sub>2</sub> after adding vit.	Per cent of increase	Conc. vit. in culture medium in $\gamma$ per 100 cc	Conc. vit. added in $\gamma$ per vessel
Thiaminless <sup>1</sup>	12.9	18.7	45	0.1	20 <sup>2</sup>
<i>p</i> -Aminobenzoicless	22.6	28.7	27	0.06–0.4	0.1–20
	24.0	43.1	56	0.1	0.2–20
	28.0	28.0	0.0	0.1	0.002–0.02
Pantothenicless	21.8	27.0	22.8	4–40	0.1–100
Pyridoxineless	19.2	26.4	37.4	0.1–0.4	0.1–20

<sup>1</sup> Culture not shaken during growth.

<sup>2</sup> Generally 20  $\gamma$  of the vitamin were added to each Warburg vessel except in the test runs on the effect of different concentrations of vitamin.

were obtained with pantothenic acid and with pyridoxine. The rate of penetration is probably not the limiting factor, either, under the conditions of the experiment, for two of the vitamins used are organic acids which should penetrate most effectively in the undissociated state (see Wyss *et al*, 1944). Dissociation is less at a low pH and most of the experiments were performed at pH 4.5–5.5. If these vitamins are directly involved in enzymes catalyzing particular reactions in respiration it might be possible to demonstrate this by testing their effects on the oxidation of a variety of substrates. It was found that wild type and mutant strains of *Neurospora*, starved for sucrose only, are able to

oxidize hydrolyzed casein or a mixture of synthetic amino acids fairly well, but not single amino acids; similarly, they oxidize glycerol, ethyl alcohol, acetic, pyruvic and lactic acids fairly well, but not malic and succinic acids. To test the effect of vitamins, the mutants must be starved for both vitamin and sucrose. However, mutant strains starved for both sucrose and vitamin did not respond with much regularity to these nutrients and often the mycelia disintegrated and the response

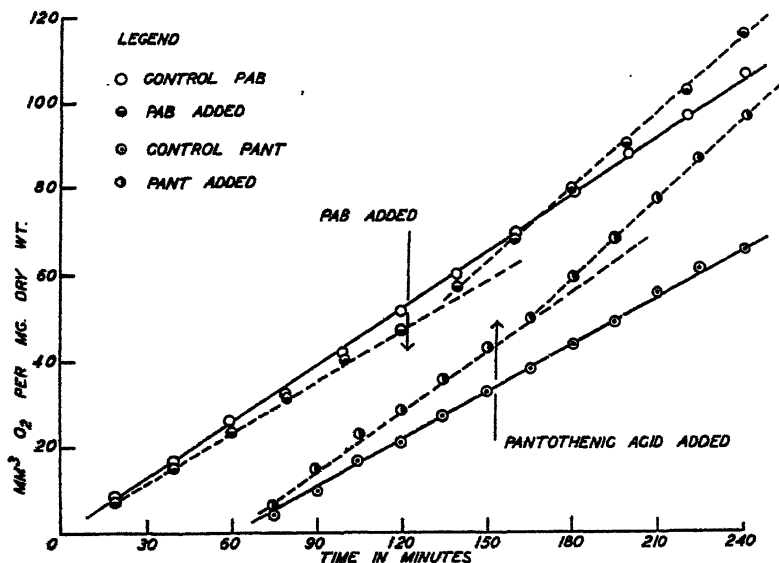


FIG. 4

Effect on Respiratory Rate of the Addition of Pantothenic Acid to the Pantothenicless Mutant and of *p*-Aminobenzoic Acid to the *p*-Aminobenzoicless Mutant When the Mutants Are Starved for the Respective Vitamins by Growth in Inadequate Vitamin.

was very feeble. This is due to the high degree of starvation required, for if a full grown mycelium is merely shaken in a medium devoid of nutrients it continues respiring much as it did in the medium, apparently on material stored in the cells, only gradually showing a decline in the rate of respiration. The exhaustion of the stored nutrients is so slow and the enfeeblement resulting from prolonged starvation of this type so great, that the study of vitamin effects on substrates other than sucrose had to be abandoned as the results were too irregular. All that

can be said from these experiments is that the oxidation of such substrates as were tested seemed to depend on the vitamin concentration in the same way as did the oxidation of sucrose. In other words, no specific relationships of substrate to vitamin were observed.

On the other hand, if the vitamins under investigation accelerate respiration indirectly by an effect on the synthesis of other vitamins concerned directly with respiratory enzymes, one might expect that increasing the other vitamins in the solution might influence the general

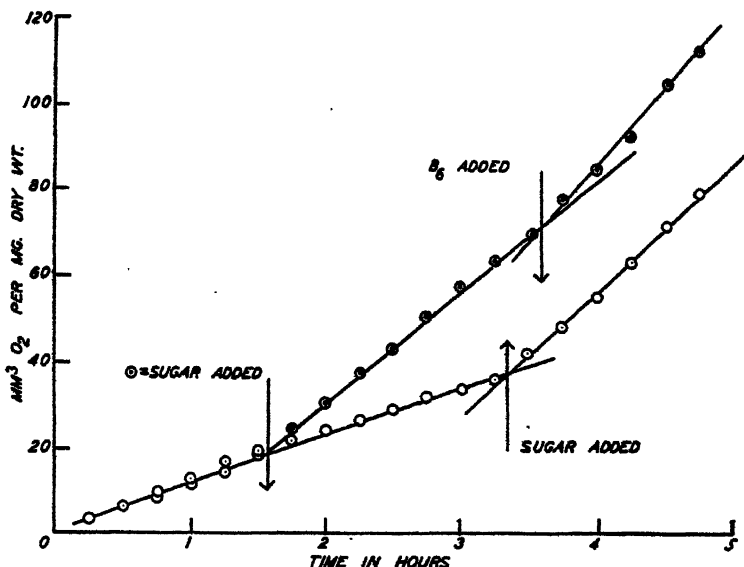


FIG. 5

Effect of Addition of Sugar and Pyridoxine to the Pyridoxineless Mutant Starved for Both Sugar and Vitamin

metabolism. It was found, however, that the total respiration was unaffected by additions of vitamins other than the one for which a given culture was starved. Also, after the addition of the deficient vitamin, additions of other vitamins of the B-complex did not further increase the rate of respiration. Any effect would, therefore, be on the incorporation of the respiratory vitamins into the enzyme systems of the cell rather than on the synthesis of the vitamins themselves.

*Experiment on Respiratory Quotients.* It seemed possible that with more definite information on the overall metabolism of *Neurospora*



under different conditions of starvation one might be able to analyze the functions of the B-vitamins under investigation. Respiratory quotients were, therefore, determined under various conditions, in some cases by the standard Warburg methods and in others with Summerson manometers.

For normal cultures of wild type or the vitamin mutants, the R.Q. values were consistently greater than unity. With the Summerson manometers values as high as 1.95 and 2.28 were obtained in two instances. During starvation for vitamins in the vitamin mutants, the values fell gradually to 1.0, but no lower values were observed in the presence of carbohydrate. When the wild type was starved for carbohydrate, however, the R.Q. fell to unity and then to less than unity, the lowest observed being 0.66, the average for five determinations being 0.75. When such starved cultures were supplied with sucrose the R.Q. returned to 1.0 in three hours.

The high R.Q. observed in normal healthy cultures could be imagined as due to the production of reduced products such as fat, alcohol and proteins. Tests were made for accumulation of fat by staining with Sudan black (Hartman, 1940). No very marked difference could be discerned between the experimental and the control cultures, except in very old cultures in which there is a greater accumulation of black-staining globules. Determinations of alcohol were made by the colorimetric method of Gibson and Blotner (1938). A 24-hour culture of *Neurospora* contained alcohol equal to 8% of the weight of the mycelium, a 48-hour culture 17%, a 72-hour culture 52% and a 96-hour culture 74%.<sup>5</sup> In the latter case, about 24% of the sucrose decomposed appeared as alcohol. Alcohol is, therefore, produced in larger quantity as the culture ages and as O<sub>2</sub> consumption and growth decrease. Calculations have indicated that in older cultures the production of fat and alcohol could account for the observed R.Q. values. However, there must be other reduced materials formed in young active cultures to account satisfactorily for the high R.Q. values observed. It seems likely that the most important reduced product in these cultures is protein.

## DISCUSSION

It is clear from this study that the rate of respiration may not always be a clear index of the state of any given system in the respiratory

<sup>5</sup> After 96 hours growth 10 mg. of the 750 mg. of sucrose supplied per 50 cc. of Fries medium still remain.

complex. This rate is due to so many factors that analysis is difficult. This is brought out clearly in studies on the effects of ultraviolet light on respiration (Giese, 1941, 1942) where the rate of exogenous respiration is seen to remain at a normal level, even when the cells have been exposed to a dosage which is sufficient to prevent their multiplication and many metabolic processes may have been impaired. In the present study it is indicated in the difficulty with which the rate of respiration of *Neurospora* is affected by the medium. It is also strikingly brought out in the "stunted" morphological mutant strains which grow at a very slow rate yet show a respiratory rate of the same order of magnitude as the rapidly growing controls.

The increase in rate of respiration in *Neurospora* on addition of B<sub>1</sub> is not surprising in view of the function of this and certain other vitamins as prosthetic groups of important respiratory enzymes (see Rosenberg, 1945). The similar results with *p*-aminobenzoic and pantothenic acids and with pyridoxin suggest that these vitamins may have similar functions in *Neurospora*. However, *Neurospora* proved rather unsatisfactory for respiratory measurements and, so far, attempts to define the exact respiratory functions of these vitamins have not been successful. It has not been possible to associate the increases in rate of respiration with specific metabolic reactions or enzymes nor to demonstrate conclusively that the effects are indirectly upon the entire enzymatic system of the cell, although the latter seems most probable from the available evidence.

Respiratory quotients appreciably lower than unity were observed in cultures starved for carbohydrate, or for carbohydrate and vitamins. These values suggest oxidation of reduced cellular materials. If the main reduced product is protein, as seems likely from other considerations, and if this is oxidized in the absence of available carbohydrate, it is understandable that protein depletion might result in the general debility of the cells so consistently observed in cases of extreme starvation, and evidenced by the progressive failure of such cells to respond to vitamin or carbohydrate additions.

The results suggest that the high R.Q. values of healthy young cultures are due primarily to assimilatory reactions, possibly the synthesis of protein. There can be very little protein or other reduced product synthesized under conditions of vitamin or carbohydrate starvation which result in R.Q. values of unity or less. The values of approximately unity found for vitamin starved cultures suggest that

there is no qualitative impairment of oxidative metabolism and that the vitamin deficiencies result in the failure of assimilatory reactions, possibly of protein synthesis.

If *p*-aminobenzoic acid, pantothenic acid and pyridoxin are concerned primarily in assimilatory processes such as protein synthesis, and are not directly involved in respiratory enzymes, how are the stimulating effects of their additions on the  $Q_{O_2}$  values to be interpreted? It seems most probable that such increases in the rate of oxygen uptake result from increases in the concentrations or activities of enzymes catalyzing reactions fairly directly involved in oxygen consumption. The evidence suggests that deficiencies for the three vitamins investigated do not result in deficiencies for other vitamins, which might be constituents of respiratory enzymes. One possibility which might be suggested is that *p*-aminobenzoic acid, pantothenic acid and pyridoxin are involved not only in overall protein synthesis, but also in the synthesis of enzyme proteins. If this were true, deficiencies in these vitamins might result in lowered respiratory enzyme concentrations and their additions would result in increased enzyme synthesis and then in an acceleration of the rate of oxygen consumption. A somewhat analogous interpretation has been suggested for the effect of biotin on respiration and ammonia utilization of *S. cerevisiae* (Winzler, Burk and du Vigneaud, 1944). Recent evidence has indicated that one of the vitamins studied in the present investigation with *Neurospora*, pyridoxine, is also involved in protein metabolism (Lepkovsky, Roboz and Haagen-Smit, 1943; Gunsalus, Bellamy and Umbreit, 1944; Schlenk and Snell, 1945).

#### SUMMARY

1. Wild type and vitamin mutant strains of *N. crassa* and *N. sitophila* have a  $Q_{O_2}$  of from 10 to 55 mm<sup>3</sup> O<sub>2</sub> per hour per mg. dry weight, the value depending on the age of the culture and the state of nutrition.
2. Morphological mutants which grow very slowly, even when supplied with a "complete" medium, respire at a rate of the same order of magnitude as the control wild type.
3. The respiratory rate of wild type starved for sucrose may fall to as low as 5 mm.<sup>3</sup>/mg. dry weight/hour and on addition of sucrose will increase on the average by 340%.
4. Cultures starved for vitamin alone show only a small decline in respiration and on addition of vitamin show an increase of from 13 to

70%. However, cultures starved too long respond only feebly to additions.

5. The respiratory rate of the vitamin mutants falls to even lower levels following starvation for both sucrose and vitamin.

6. The respiratory quotient is generally greater than one in the healthy cultures, close to one for vitamin-deficient cultures, and less than one in cultures starved for carbohydrate. Alcohol and fats are produced in amounts inadequate to account for the high respiratory quotient of vigorous cultures.

7. The increases in respiration produced by their addition to properly starved mutant strains indicate that *p*-aminobenzoic acid, pantothenic acid and pyridoxine may have respiratory functions in *Neurospora*. It is suggested that these functions may be indirectly concerned with respiratory enzymes, possibly through the effects of these vitamins on protein synthesis.

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# Sulfanilamide and Respiration of *Neurospora*<sup>1</sup>

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## INTRODUCTION

The basis of the anti-bacterial action of sulfanilamide is still in question in spite of the tremendous volume of research in this field (Henry, 1943). Sulfanilamide has no effect on respiration of most micro-organisms. However, sulfonamides have been reported to inhibit the action of certain enzymes (Sevag and Shelbourne, 1942; Sevag, Shelbourne and Ibsen, 1942) as well as overall respiration (Wyss, *et al.*, 1942; Sevag, *et al.*, 1945a, 1945b) and especially assimilation in *E. coli* (Clifton and Loewinger, 1943). The inhibition of the growth of *Neurospora* by sulfanilamide (Tatum and Beadle, 1942) is counteracted by *p*-aminobenzoic acid which is in accord with the Woods-Fildes hypothesis of a specific relationship between sulfanilamide and *p*-aminobenzoic acid (Woods, 1940; Woods and Fildes, 1940). The problem then was to determine whether the growth retardation of *Neurospora* was the result of an inhibition of respiratory activity. It seemed particularly desirable to test *Neurospora* since a *p*-aminobenzoicless mutant, the growth of which is a function of the *p*-aminobenzoate supplied, was available for the study.

## EXPERIMENTAL

*Cultures and Methods.* *Neurospora crassa* 1A (wild type) and *N. crassa* 1633 *p*-aminobenzoicless (Tatum and Beadle, 1942) were used. Respiratory measurements were made by standard Barcroft-Warburg techniques using cultures of *Neurospora* grown under conditions described elsewhere (Giese and Tatum, 1945). Growth experiments

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were carried out in synthetic medium (Horowitz and Beadle, 1943) using 25 ml. of medium in 250 ml. flasks, incubated at 25°C.

*Effect of Sulfanilamide on Respiration.* When the wild type mycelium of *Neurospora* was exposed to sulfanilamide (or sulfapyridine or sulfathiazole) in a variety of concentrations up to saturation, there was no effect on oxygen uptake over periods up to four hours after addition of the drug. The oxygen consumption of wild type *Neurospora* supplied with limiting concentrations of sugar also was not significantly different from that of controls. Similarly conducted experiments with the *p*-aminobenzoicless mutant strain, grown in either adequate or sub-optimal supplies of *p*-aminobenzoic acid also failed to show any effect on respiration as did analogous experiments with the thiaminless, pyridoxineless and pantothenicless mutants.

It was thought that the absence of respiratory inhibition might be due to the failure of sulfanilamide to penetrate the cells at the pH used. Since pH affects the action of sulfanilamide in certain cases (Schmelkes, Wyss, Marles, Ludwig and Strandkov, 1942), tests with both strains of *Neurospora* were repeated at a variety of pH values. There was no effect of sulfanilamide on respiration over a pH range of 4 to 8.

Since, in these experiments, respiration was followed for only a few hours after the addition of sulfanilamide, it seemed possible that an inhibition of respiration might be found after longer treatment. Wild type cultures grown for periods up to three days in different concentrations of sulfanilamide respired at approximately the same rate as normal cultures, in spite of the inhibition of growth observed in the higher concentrations. Finally, cultures depleted of carbohydrate reserves by growth in medium containing only 0.1% sucrose were exposed to sulfanilamide for periods up to 24 hours. The respiratory responses of these cultures to carbohydrate additions were then compared with those of control starved cultures. There were no significant differences in the absolute respiratory rates of the two cultures on addition of sucrose. However, it was observed that the increase in  $Q_{O_2}$  in per cent of the basal endogenous  $Q_{O_2}$  was significantly greater in some cultures treated with sulfanilamide. This was true with a variety of substrates as shown in Table I. There were no differential variations in the  $Q_{O_2}$  values of specific substrates which could be correlated with the sulfanilamide treatment. The greater per cent increases of the sulfanilamide cultures were probably due in part to the lower basal rates of these cultures, since the absolute  $Q_{O_2}$  values after substrate additions were about the

TABLE I

*Effect of Sulfanilamide on Oxygen Consumption of Carbohydrate Starved Cultures*

Figures in the body of the table give the per cent increases in consumption over the endogenous rate on addition of other various nutrients.

Nutrient Added	Control		Sulfa-poisoned <sup>2</sup>	
	No. 81	No. 83	No. 81	No. 83
Sucrose.....	62	12	144	100
Pyruvate.....	25	11	31	20
Glycerol.....	35	33	100	100
Acetic acid.....	110	86	225	175
Lactic acid.....	0	11	38	20
Amino acids <sup>1</sup> .....	27	44	100	25
Endogenous QO <sub>2</sub> .....	18-55	16-24	16-21	12-16

<sup>1</sup> Hydrolyzed casein.

<sup>2</sup> Treated with sulfanilamide (50 mg. per 50 ml. medium) for 24 hours before respiratory measurements.

same in both types of cultures. The possibly significant observation was made that cultures treated with sulfanilamide in the absence of carbohydrate remained in better condition physiologically than those without sulfanilamide, and showed less tendency to disintegration on prolonged carbohydrate starvation. If this deterioration is due to protein or enzyme destruction or oxidation (see Giese and Tatum, 1945), these processes may be slowed down by sulfanilamide.

If the growth effect of *p*-aminobenzoic acid on *Neurospora* (Tatum and Beadle, 1942) is due to its participation in assimilatory or syn-

TABLE II

*Growth Inhibition by Sulfanilamide <sup>1</sup>*

Age of culture at sulfanilamide addition	Wt. of culture at addition	Growth period measured	Growth as per cent of normal in the following concentrations of sulfanilamide (mg. per 50 ml.)					Final Wt. of control
			5	10	25	50	100	
(hours)	(mg.) <sup>2</sup>	(hrs. after inoculation)						(mg.)
24	1.6	24-48	27.4	22.4	26.5	29.2	21.5	27.4
24	1.6	48-96	44.6	34.2	18.1	19.2	21.7	38.6
48	23.2	48-96	—	88.4	77.5	63.5	35.1	43.8

<sup>1</sup> Strain 1633 (*p*-aminobenzoicless) with 1γ PAB per 25 ml. culture in a 250 ml. Erlenmeyer flask grown at 25°C. on a shaker.

<sup>2</sup> Dry weight, average of two series.



thetic reactions in protein metabolism as suggested by Giese and Tatum (1945), and if sulfanilamide directly antagonizes *p*-aminobenzoic activity, the action of sulfanilamide may involve the retardation or slowing of reactions concerned with both protein synthesis and breakdown (see Kohn and Harris, 1941, 1943). Both types of reactions must be involved in protein synthesis. There is an increasing body of evidence that cellu-

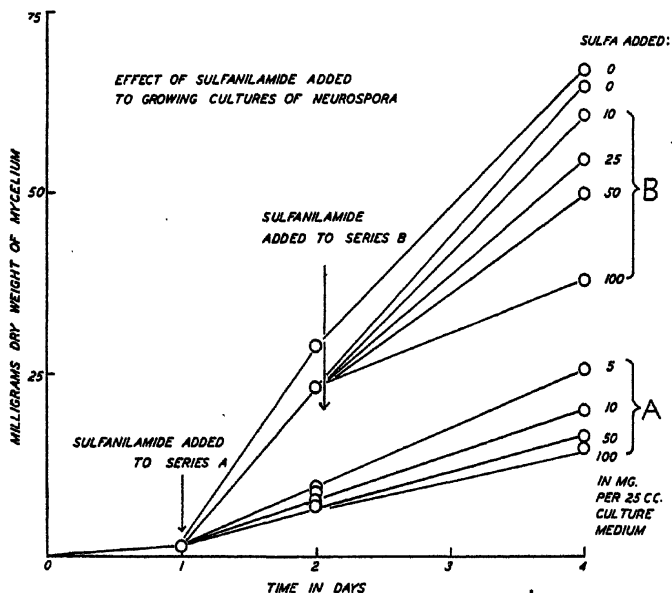


FIG. 1

Effect of Sulfanilamide Added to a Growing Culture of *Neurospora*, *p*-Aminobenzoicless Strain (No. 1633) Grown on 0.5  $\gamma$  PAB per 25 ml. Medium.

Note the much greater effect of even low concentrations added to the one-day-old culture.

lar proteins are in a continual state of change, or dynamic state (see Schoenheimer, 1942). These concepts and the suggested roles of *p*-aminobenzoic acid and sulfanilamide in growth and metabolism of *Neurospora* would necessitate a qualitative change in metabolism in *p*-aminobenzoic deficiency or in the presence of sulfanilamide. This is perhaps supported by the R.Q. of around 1.0 observed with *p*-amino-

benzoic deficient cultures (Giese and Tatum, 1945) and by the fact that R.Q. values of from 1.01 to 1.12 have been obtained with wild type cultures grown in the presence of sulfanilamide.

*Effect of Sulfanilamide on Growth.* In an attempt to further define the effects of sulfanilamide on growth of *Neurospora*, a number of growth

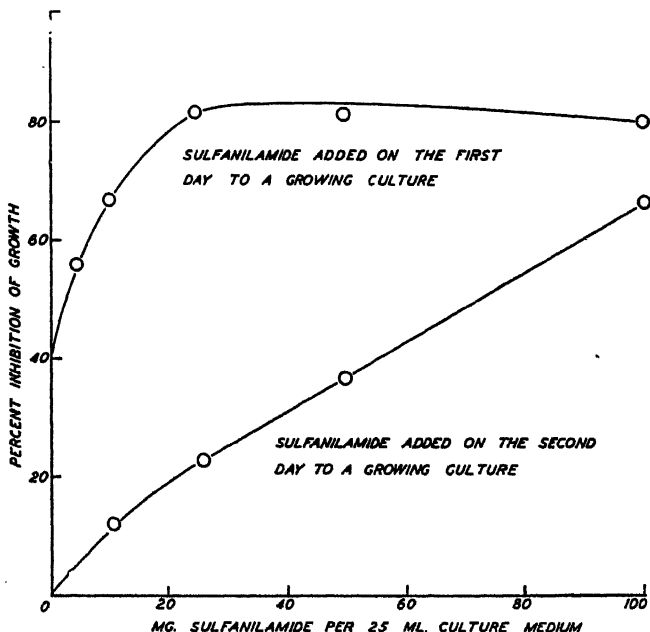


FIG. 2

Per Cent Inhibition of Growth of *Neurospora* (Strain No. 1633) Grown on 0.5  $\gamma$  PAB per 25 ml. by Sulfanilamide Added at Different Times During Growth.

Effects measured between the second and fourth days of culture. Note the much greater effect on the younger culture. Compare with the value of 8 mg. per 26 cc. of sulfanilamide required to inhibit germination of conidiospores in liquid medium (Tatum and Beadle, 1942).

studies were made. It has previously been shown that concentrations of sulfanilamide of 10 mg. per 15 ml. will completely inhibit germination of *Neurospora* conidia on solid medium, as will concentrations of 8 mg. per 25 ml. in liquid medium (Tatum and Beadle, 1942).

In order to test the relation of sulfanilamide to growth under conditions more comparable to those used in the respiratory studies, wild

type cultures were grown in liquid media for different periods of time before sulfanilamide additions. Subsequent growth measurements are given in Table II. The following conclusions seem justified: (1) a mycelium growing in liquid medium is resistant to much higher concentrations (up to 50 mg. per 25 ml.) of sulfanilamide than will inhibit conidial germination or growth on solid medium; (2) the degree of inhibition is reciprocally related to the weight of the mycelium (which is a function of the age of the culture) at the time of sulfanilamide additions; (3) the inhibitory effects of lower sulfanilamide concentrations is overcome after a period of time; (4) the growth is an inverse function of sulfanilamide concentration up to a concentration of about 25 mg. per 25 ml., which inhibits growth to about 20% of the control. However, higher concentrations, up to 100 mg. per 25 ml., do not further inhibit growth, as shown in Fig. 1.

#### DISCUSSION

Since sulfanilamide fails to reduce the  $Q_{O_2}$  of *Neurospora*, even in very high concentrations, its inhibition of growth of this organism cannot be due to changes in oxygen consumption and may be due to effects on reactions not directly concerned in overall oxygen uptake. As *p*-aminobenzoic acid reverses the growth-inhibiting action of sulfanilamide, a specific relation to *p*-aminobenzoic acid is suggested. Respiration of cultures of the mutant grown in minimal or insufficient *p*-aminobenzoic acid is not affected by additions of sulfanilamide. However, addition of the vitamin to such deficient cultures is followed by an increase in respiration (Giese and Tatum, 1945). It is, therefore, possible that *p*-aminobenzoic acid has more than one effect on metabolism and that only some synthetic activity leading to cell growth is inhibited by sulfanilamide. The facts that R.Q. values of about 1.0 are found in cultures grown in sulfanilamide and in *p*-aminobenzoic-deficient cultures of the mutant strain, whereas values well over 1.0 and in the extreme approaching 2.0 are found for healthy actively growing cultures of the mold may support this view, since the high R.Q. of active cultures seems best explained by protein synthesis (Giese and Tatum, 1945). The fall in the R.Q. in aged, starving or poisoned cultures is presumably due to the decline in the rate of synthesis. Thus a qualitative change in the metabolism of *Neurospora* following poisoning with sulfanilamide seems indicated even though quantitative changes in  $Q_{O_2}$

are not observed after addition of the poison. A similar role of sulfanilamide has been suggested by various studies with micro-organisms (Clifton and Loewinger, 1943; Kohn and Harris, 1941 a, b, 1943; Harris and Kohn, 1940, 1941, 1943; von Euler, 1942; Anderson, Pilgrim and Elvehjem, 1944; Dorfman and Koser, 1942).

The difference in effects of sulfanilamide on germination of conidia (Tatum and Beadle, 1942) and on growth of *Neurospora* mycelium may be satisfactorily explained on the basis of the above concept of the action of the poison. The conidia appear to be very sensitive to sulfanilamide and are easily prevented from germinating, whereas cultures already actively growing can be inhibited only to the extent of about 80% in the presence of 100 mg. of sulfanilamide per 25 ml. of culture fluid. In conidia the enzymes are presumably only present in very low concentrations and active synthesis is required for growth. In growing cultures enzymes are already present in larger amounts. Therefore, the greater sensitivity of conidia is to be expected. This would be somewhat analogous to the apparent need of carboxylase for the germination of *Neurospora tetrasperma* ascospores (Goddard and Smith, 1938).

The results stand in contrast to the effects of iodoacetate since the threshold concentrations of iodoacetate for inhibition of germination of conidia and of growth of mycelium are similar (Ryan, Tatum and Giese, 1944). Iodoacetic acid also has a definite inhibitory action on respiration as well as on growth of *Neurospora* and this inhibition is released by succinic acid. The mere retardation of growth of mycelia of *Neurospora*, in contrast to the complete inhibition of growth of conidia, by sulfanilamide suggests that in growing *Neurospora* certain reactions essential for growth are resistant to sulfanilamide since growth could not be reduced to lower than about 20% of normal. These resistant reactions may be qualitatively different from the susceptible reactions responsible for 80% of normal growth of the mycelium. Since sugar, even in limiting amounts, is respired to about the same degree by poisoned cultures and controls, there seems to be no interference by sulfanilamide with gross uptake or respiration of this nutrient. The respiration of other nutrients is also not reduced in poisoned cultures (Table I). The key germination processes, on the other hand, appear to be 100% susceptible to sulfanilamide inhibition. This may be due to qualitative differences in these key reactions which occur at germination.

## SUMMARY

1. The rate of respiration of *Neurospora* is not affected by sulfanilamide even when the medium is saturated with the poison. This is true of healthy and starved cultures of either the wild type or the *p*-aminobenzoicless strain. Deficiency of vitamin in the latter strain does not render its respiration more sensitive to sulfanilamide.

2. The oxidation of sucrose starved mycelium is not altered by the simultaneous addition of sulfanilamide. However, mycelia starved in the presence of sulfanilamide, when supplied with various nutrients, including sucrose, respond even better than do the controls. This suggests that sulfanilamide may have a preservative effect on the respiratory structure of the cell. This effect may result from inhibition of protein (enzyme) destruction during starvation.

3. The growth of cultures of *Neurospora* actively growing at the time of addition of sulfanilamide is inhibited only to an extent of 80%.

4. The results can be interpreted on the Woods-Wildes hypothesis only if it is assumed that sulfanilamide and its analogues affect synthetic reactions. Qualitative changes in metabolism which presumably follow sulfanilamide interference with the synthetic processes resulting in growth do not bring about quantitative changes in total oxygen consumption.

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# The Photochemical Production of Oxygen and Hydrogen Ion by Isolated Chloroplasts <sup>1</sup>

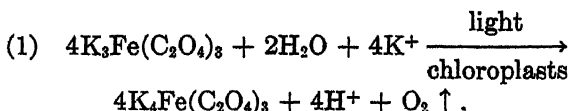
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## INTRODUCTION

In 1937 and 1939 R. Hill (1, 2) reported that when isolated chloroplasts were suspended in an aqueous extract of acetone-treated yeast or leaves and illuminated, O<sub>2</sub> was evolved. In the yeast extract the active principle was found to be a ferric salt of some organic acid or acids. Of several ferric salts tried as substitutes, potassium ferric oxalate resulted in the highest rate of O<sub>2</sub> evolution. Also reported was the fact that simultaneously with the O<sub>2</sub> evolution there was a reduction of the potassium ferric oxalate, and that the volume of oxygen produced per unit of ferric salt corresponded closely with the theoretical amount that would be obtained from the following reaction, which has been designated as the Hill reaction by French, Newcomb and Anson (3):



Up to the present, three methods of measuring the activity of chloroplast suspensions for producing oxygen in the above manner have been reported. These are: (a) spectrophotometric determination of the rate of oxyhemoglobin formation when the oxygen was evolved in a solution of muscle hemoglobin (1, 2); (b) measurement of the ferrous iron produced during the Hill reaction by its action on methemoglobin which it reduces to hemoglobin. The latter was measured spectroscopi-

<sup>1</sup> Part of the equipment used in this work was purchased with a grant from the Graduate School of the University of Minnesota.

<sup>2</sup> The experiments reported here will be submitted in partial fulfillment of the requirements for the Ph.D. degree from the University of Minnesota.



cally as oxyhemoglobin (4); (c) manometric measurement of the rate of oxygen evolution when potassium ferricyanide was used as the oxidizing agent (5). The latter method allowed oxygen to accumulate to such pressures as would have caused reoxidation of the potassium ferrous oxalate by oxygen in the hemoglobin methods.

In this paper we are presenting a constant pH titration method with which the rate of the reaction is measured by neutralizing the acid produced from the reduction of potassium ferric oxalate.

By means of the manometric and titration methods the effects of different variables on the rate of the reaction were investigated and are reported here.

## METHODS

The methods and the apparatus used in the isolation of the chloroplasts and in the measurement of the amount of chlorophyll in the chloroplast suspensions were the same as those used by French and Anson (6). Fresh market spinach was used throughout as a source of chloroplasts. The composition of the reaction medium, referred to hereafter as Hill's solution, was as follows:

$K_3Fe(CN)_6$	0.02 M
$Fe(NH_4)(SO_4)_2$	0.01 M
$K_2C_2O_4$	0.50 M
Sucrose	0.20 M

Sodium sorbitol borate (0.17 M) was used as the buffer in the manometric measurements.

### 1. Measurement of the reaction by titration

As seen from Equation 1, in addition to the production of one mole of oxygen, four moles of acid should be produced. In view of data presented later we believe this stoichiometric relationship to be valid also in the presence of potassium ferricyanide and we have used the acid production as a means of measuring the rate of the reaction. The method was designed primarily to make possible rapid determinations of the activity of various chloroplast preparations. Measurements have been completed within ten minutes after isolation of the chloroplasts. The unit used for comparing the rates of  $H^+$  production from different chloroplast suspensions was

$$(2) \quad Q_{H^+}^{ch} = \frac{\text{cu. mm. } H^+}{\text{hrs.} \times \text{mg. chlorophyll} \times 4}$$

$$= \frac{(\text{Rate of base consumption in ml./hr.}) \times (\text{Normality of base}) \times 22.4 \times 10^4}{\text{mg. chlorophyll} \times 4}$$

The factor 4 in the denominator is used on the basis of Equation 1 to make  $Q_{H^+}^{ch}$  directly comparable with  $Q_{O_2}^{ch}$ .

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$$* Q_{O_2}^{ch} = \frac{\text{cu. mm. } O_2}{\text{hrs.} \times \text{mg. chlorophyll}} \quad (\text{Reference 6})$$

To make these measurements, 10 ml. of Hill's solution were placed in a 50 ml beaker around which cold water was circulated in a glass bottomed chamber. The beaker contained the extension electrodes of a Beckman pH meter, a thermometer, a motor driven glass stirrer, and the tip of a 5 ml. automatic burette. The burette, graduated to 0.01 ml., was surrounded by a glass jacket through which the cold water circulated after having passed through the glass-bottomed chamber. When the Hill's solution came to the desired temperature, the chloroplast suspension was added and the pH was rapidly adjusted with 0.1  $N$   $H_2SO_4$  or KOH. The beaker was illuminated from below by a 1000 watt tungsten bulb with a parabolic reflector which provided about 4,500 foot candles filtered through 4 inches of water below the beaker. A stopwatch was then started, after which 0.1–0.3 ml. of weak KOH

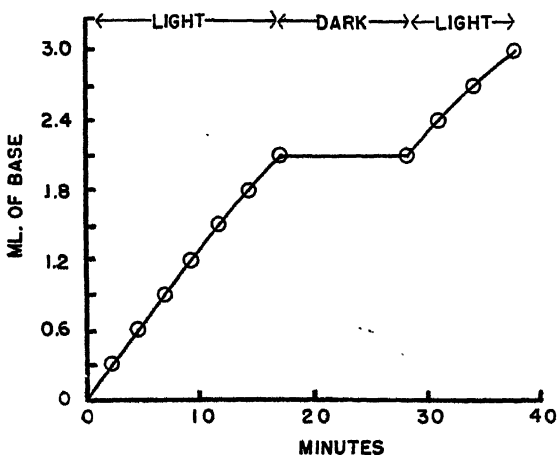


FIG. 1

The Production of  $H^+$  by Illuminated Chloroplasts in 10 cc. of Hill's Solution

The amount of 0.014  $N$  KOH required to keep the pH at 6.5 is plotted against time. The temperature was 10°C. and the chloroplast concentration was equivalent to 0.40 mg. chlorophyll per 10 ml. Hill's solution

(0.015 or 0.005  $N$ ) was added from the burette. This caused a very slight increase in pH, and the time required for the pH to return to the original value was recorded. Another 0.1–0.3 ml. portion of the base was immediately added and the procedure repeated as long as necessary. Measurements were usually continued for 5–10 minutes. We were thus able to obtain a plot of the ml. of base consumed against elapsed time of illumination from which the initial rate of  $H^+$  production was obtained graphically.

Acid production stopped when the light was turned off as shown in Fig. 1, indicating that thermal oxidation of chloroplast constituents by the ferricyanide could not have been responsible for the observed acid production during the illumination period. The sensitivity of this method depends upon the amount of pH change

produced in the mixture of chloroplasts and Hill's solution when a given amount of acid or base is added. The sensitivity is greatest near neutrality but it has been found to be adequate in the range from pH 5.5 to pH 8.0. The titration curves of a chloroplast suspension and of Hill's solution at the concentrations used for most of this work are given in Fig. 2. It is evident that the chloroplast material is responsible for only a very small part of the buffering action.

## 2. Measurement of the Oxygen Evolution

Two ml. of Hill's solution made up 1.5 times the concentrations listed above and 0.5 ml. of 1 *M* sodium sorbitol borate were added to the main space of a double-

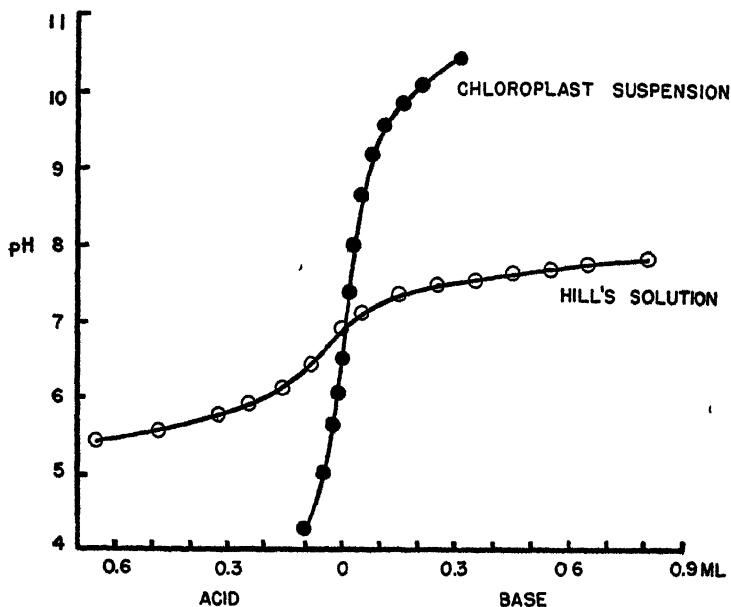


FIG. 2

Titration Curves of 10 ml. of Hill's Solution, and of 2 ml. of a Chloroplast Suspension Containing 0.560 cu. mm. of Chloroplasts with 0.1 *N* HCl and 0.1 *N* KOH

armed Warburg vessel. One-half ml. of chloroplast suspension was placed in one sidearm, and 0.3 ml. of 10% KOH was placed in the other to absorb  $\text{CO}_2$ . The vessels were immersed in a thermostat tank at 15°C. and rocked for five minutes while nitrogen was being introduced. This was followed by a dark equilibration period of approximately fifteen minutes, at the end of which time, the chloroplast suspension was tipped into the main space of the vessel. The vessels were then illuminated by light provided by one 40 watt frosted "Lumiline" bulb, eighteen inches long and located one inch below the vessels. The light intensity on the bottom of the vessels obtained from this system was approximately 250 foot candles. The first reading

was taken at the end of ten minutes illumination and subsequent readings were taken at five minute intervals for thirty-five minutes more. The initial rate of the oxygen evolution in cubic millimeters per minute was determined graphically, and converted to the rate in cubic millimeters per hour per milligram of chlorophyll, (i.e.,  $Q_{O_2}^{ch}$ ).

To rule out such variables as differences in the areas of the bottoms of the vessels exposed to the illuminating system, a series of experiments using five manometer-vessel combinations at a time were made with conditions as nearly identical as possible. Only those combinations which varied 5% or less in rate at fifteen minutes were used for subsequent experiments.

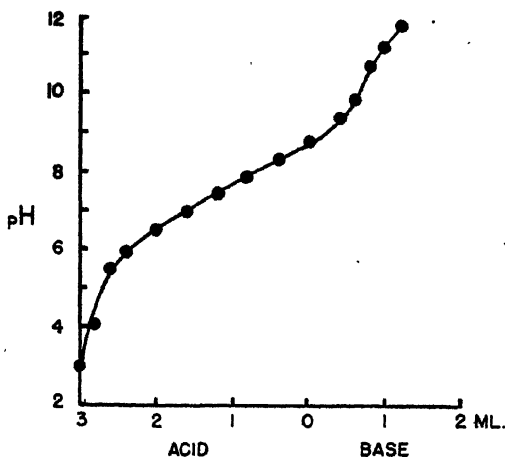


FIG. 3

Titration Curve of 250 ml. of 0.002 *M* Sodium Sorbitol Borate  
with 2 *N*  $H_2SO_4$  and 2 *N*  $KOH$

A solution of 1 *M* sodium sorbitol borate buffer was prepared from the approximately 3.3 *M* Atlas Powder Company<sup>3</sup> product. One disadvantage of using this reagent as a buffer was that it buffered only at constant volume, the pH increasing considerably on dilution. This was compensated for by adding either 25% sulfuric acid or 50% potassium hydroxide to the stock solution in such concentration as would result in the desired final pH for the vessel contents.

To obtain a titration curve that would not be complicated by the dilution effect due to addition of the titrating acid or base, 250 ml. of a 0.002 *M* solution were titrated with 2 *N* sulfuric acid and 2 *N* sodium hydroxide. The curve is shown in Fig. 3, and is typical of that obtained from the titration of a boric acid solution to which such substances as glycerol and mannitol have been added (7). The fact that

<sup>3</sup> We wish to thank Mr. H. C. Speel of the Atlas Powder Company, Wilmington, Del., for the samples of sodium sorbitol borate.

it buffered from pH 5.5 to pH 9.0 and did not precipitate insoluble iron salts made it a suitable reagent for the usual physiological range. Measurements of the pH of the buffered Hill's solution-chloroplast mixture before and after a forty-five minute period of illumination in the Warburg vessels showed but a few hundredths of a pH unit decrease. All pH readings were made with a Beckman glass electrode pH meter at 20°C.

## RESULTS

### 1. *The Effect of Light on the Production of Acid from Hill's Solution Without Chloroplasts*

To make sure that the observed acid production was due solely to the photochemical reduction of potassium ferric oxalate by the chloroplasts, the effects of light on the various constituents of Hill's solution, alone and in combination, were determined.

TABLE I  
*The Production of Gas from Hill's Solution*  
White light of 250 f.c.; temperature 15°C.

Sidearm	Main space	Atmosphere	Cu. mm. of gas produced or consumed in 1 hour	
			Calculated as O <sub>2</sub>	Calculated as CO <sub>2</sub>
10% KOH	Hill's solution	N <sub>2</sub>	+ 1 <sup>1</sup>	—
—	Hill's solution	N <sub>2</sub>	—	+ 17
10% KOH	Hill's solution plus boiled chloroplasts <sup>2</sup>	air	- 7	—
—	Hill's solution plus boiled chloroplasts <sup>2</sup>	N <sub>2</sub>	—	+ 9

<sup>1</sup> Not significant since the readings are accurate only to  $\pm 1.0$  cu. mm.

<sup>2</sup> The chloroplast concentration was equivalent to 0.3 mg. of chlorophyll per vessel.

Hill's solution when illuminated by white light (4500 foot candles) produced acid. This, presumably, was due to photoreduction of ferric oxalate, since illuminating a solution of potassium ferric oxalate caused the production of acid and of ferrous iron as shown by a strong positive test with *o*-phenanthroline after illumination. Acid production by Hill's solution was eliminated by filtering the light through a Corning No. 246 red filter which removed wave lengths below 568 m $\mu$ . No ferrous iron or acid was observed after a ten minute period of illumination of a solution of potassium ferric oxalate by the red light. When a solution of potassium ferricyanide was illuminated by the unfiltered

light, it very rapidly became basic. This effect was also eliminated by means of the same filter. The presence of inactive (boiled) chloroplasts or a lowering of the light intensity caused reduction of the rate of acid production by Hill's solution. No pH change occurred when boiled chloroplasts in Hill's solution were illuminated by the filtered light.

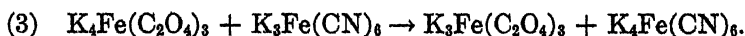
The possibility of gas production from Hill's solution when illuminated by white light of *ca.* 250 f.c. as used for the manometric experiments was also investigated. The results are given in Table I, and show that a nitrogen atmosphere and 10% KOH in the sidearm are necessary if the observed volume changes are to result entirely from oxygen evolution by the chloroplasts.

No gas exchange measurements were made at the higher intensity, which was used only with the titration assembly. Ferric oxalate is known to evolve CO<sub>2</sub> and CO when illuminated with blue or ultraviolet light. This is evidently an entirely different type of reaction from the photoreduction of ferric oxalate by illuminated chloroplasts which results in the simultaneous evolution of oxygen.

## 2. *The Stoichiometric Relations Between O<sub>2</sub>, H<sup>+</sup>, and Ferricyanide*

The relationships expressed by Equation 1 were found by Hill, and by Hill and Scarisbrick (2, 4) to be valid by their hemoglobin and methemoglobin methods. For the two methods used in this paper, involving the presence of ferricyanide, nothing has been presented concerning the agreement of the observed amounts of oxygen and acid produced with the amounts expected from Equation 1.

The reaction between potassium ferrous oxalate and potassium ferricyanide is presumed to be:



This means that the total amounts of the oxygen and acid produced by the reaction of Equation 1 should be quantitatively related to the amount of ferricyanide present provided the ferricyanide concentration is small and the reaction is allowed to run to completion. This assumption should be valid if the rate of photooxidation is low, and if there are present only negligible amounts of substances capable of reducing ferricyanide but not involved in the Hill reaction. Using Hill's solution modified to contain  $15 \times 10^{-6}$  moles of ferricyanide per vessel, the theoretical volume of oxygen that should have been evolved was 84

mm.<sup>3</sup> With nitrogen atmospheres the volumes obtained from five separate determinations were 78, 78, 76, 78, and 80 mm.<sup>3</sup>, respectively, giving an average of 92.9% of the theoretical volume. Further illumination of these vessels showed a slow uptake of gas, presumably due to a slow rate of photooxidation. With an air atmosphere the average yield was 72 mm.<sup>3</sup>, and further illumination caused a very rapid decrease of gas volume due to the uptake of oxygen resulting from photooxidation as shown in Fig. 4.

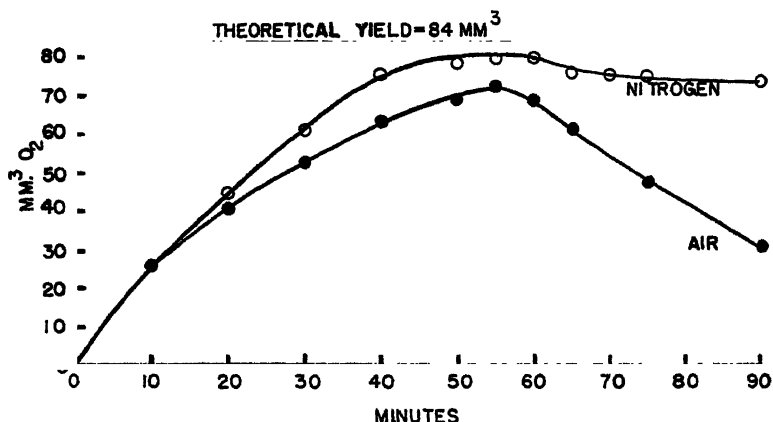


FIG. 4

The Influence of Nitrogen and Air Atmospheres on the Observed Oxygen Production from Hill's Solution Containing a Limiting Amount ( $15 \times 10^{-6}$  moles) of Ferricyanide  
pH 7.0, 13°C., 0.40 mg. of chlorophyll per vessel

It is evident that the manometric measurement of the oxygen evolution was complicated by a simultaneous photooxidation which resulted in oxygen uptake, and to reduce this effect to a minimum an inert atmosphere such as nitrogen must be used.

In the titration experiments the agreement between the amount of acid produced with the amount of ferricyanide present was close to the theoretical. The solution used contained  $6.0 \times 10^{-6}$  moles of ferricyanide, and 3.05 ml. of the 0.0197 N KOH used should have been consumed according to Equation 1. The volumes used in five separate determinations were: 2.99, 3.22, 2.90, 2.92 and 3.08 ml., respectively, making an average of 3.02 ml. or 99.0% of the theoretical amount.

### 3. The Effect of Temperature

Because of the ease of operation, it was possible, by means of the titration method, to make several successive determinations in a short time with aliquots of the same chloroplast suspension. Therefore, the titration method was chosen in preference to the manometric method for obtaining reaction data at various temperatures. However, in the

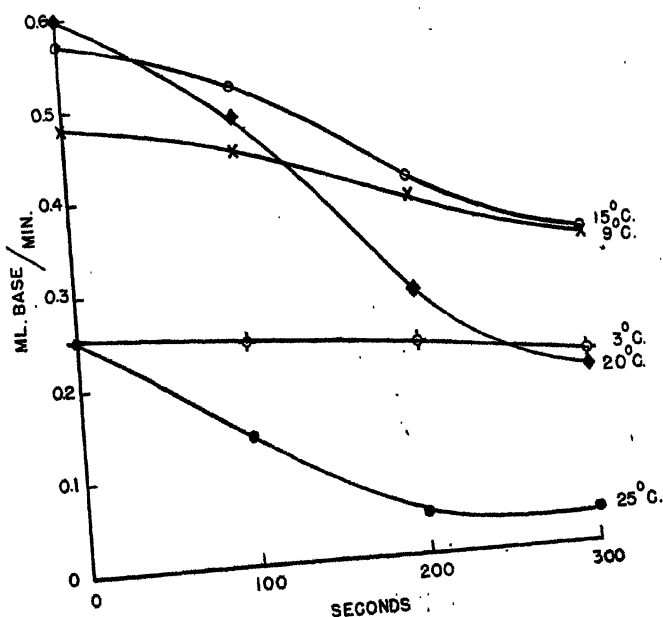


FIG. 5

The Effect of Temperature on the Rate of H<sup>+</sup> Production  
The chloroplasts were in Hill's solution 3 minutes prior to illumination.  
pH 6.8, 0.005 N KOH, 0.38 mg. of chlorophyll

cases where the effect of a variable was studied using this method, the loss of activity of the suspension on storage had to be taken into account before comparisons of the data from varied conditions could be made. When the suspension was stored at 0°C. the loss in activity was linear over a period of a few hours. This was in agreement with the findings of Hill and Scarisbrick (4). Consequently, adjustments



for the loss in activity could easily be made and the results obtained from determinations made at different times could be compared as if no loss in activity had occurred.

The rates of acid production were determined at 3°, 9°, 15°, 20° and 25°C. with aliquots from a single stock suspension. Three minutes were allowed to elapse after the addition of the chloroplasts to Hill's solution. This was done to permit final adjustments of the pH and the temperature. The results are given in Fig 5. and show that increasing

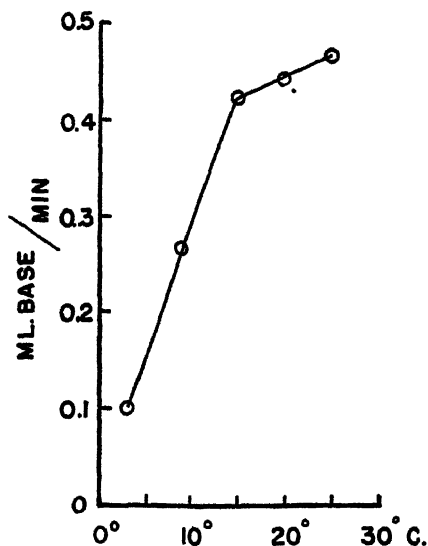


FIG. 6

The Effect of Temperature on the Rate of  $H^+$  Production at Zero Exposure of the Chloroplasts to Hill's Solution  
pH 7.08, 0.005 *N* KOH, 0.19 mg. of chlorophyll

the temperature over the range 3-20°C. caused an increase in the rate of the Hill reaction, at the same time causing an increase of the rate of the inactivation of the chloroplasts. The chloroplasts in Hill's solution were most stable at 3°C. Because of the increase of the rate of inactivation of the chloroplasts with increasing temperature it was necessary, therefore, to determine the rate of  $H^+$  production at zero exposure of the chloroplasts to Hill's solution. This was done by adjusting the pH. of Hill's solution so that pH 7.08 was obtained when 1 ml. of suspension

was added. The rates obtained plotted against temperature are shown in Fig. 6, from which it is clear that the velocity of the Hill reaction increases with temperature up to 25°C., the highest temperature used. Because of the very rapid inactivation the absolute initial rates at 20°C. and 25°C. were difficult to obtain and were very probably higher than are indicated in Fig. 6. The effect of temperature on the stability of the chloroplasts in Hill's solution is shown in Fig. 7, in which the reciprocals of the times at which the initial rate of  $H^+$  production were reduced 50% are plotted against temperature. The temperature coefficient of the Hill reaction was approximately 3.5 for the interval

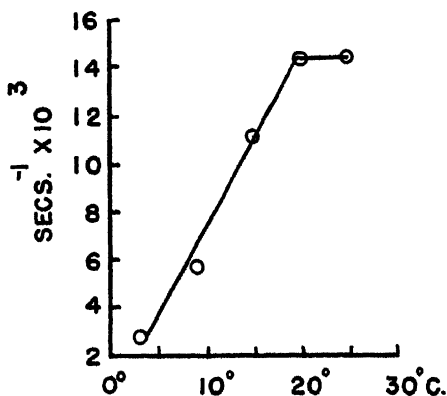


FIG. 7

The Effect of Temperature on the Rate of the Inactivation of Illuminated Chloroplasts in Hill's Solution

The reciprocal of the time required for the suspension to lose one-half of its initial activity is plotted against the temperature

3-15°C. For the same interval the temperature coefficient for the inactivation of the chloroplasts was about 3.9. The low value of  $Q_{10}$  for the inactivation could be interpreted as indicating the exhaustion of some necessary reactant within the chloroplasts rather than the denaturation of an enzyme.

#### 4. The Effect of pH

(a) *Titration Measurements:* In one set of experiments carried out at 10°C. the pH maximum was near pH 7.0. However, because of the above mentioned influence of temperature on the rate of inactivation

of the chloroplasts, these measurements are probably somewhat inaccurate, particularly at the higher pH values. Therefore, the pH curve was also determined at 3°C., the lowest temperature obtainable with our apparatus. At this temperature the maximum rate of  $H^+$  production was obtained near pH 7.60, as is shown in Fig. 8. The effect of pH on the stability of chloroplasts in Hill's solution at 3°C. can be seen in the same figure, the lower curve of which was obtained from the rates at 200 seconds of illumination (320 seconds after the chloroplasts were

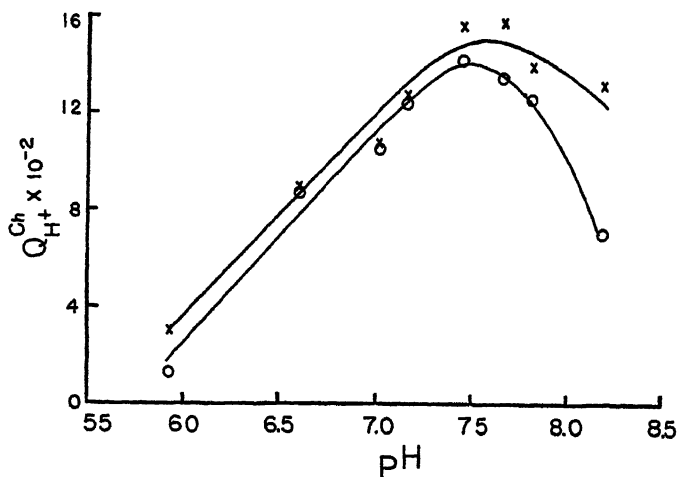


FIG. 8

Crosses: The Initial Rate of  $H^+$  Production of Illuminated Chloroplasts in Hill's Solution at Various pH Values. Circles: The Rate of Acid Production After the Chloroplasts Had Been in the Hill's Solution for 320 Seconds  
3°C., 0.25 mg. of chlorophyll

added to Hill's solution). Above pH 7.2 the chloroplasts were more rapidly inactivated the higher the pH.

(b) *Manometric Measurements*: For these experiments the concentration of the chloroplast suspensions prepared on different days was adjusted to be equivalent to 0.28 mg. of chlorophyll per vessel. Suspensions prepared from different batches of spinach give different rates per unit of chlorophyll concentration, and to compensate for this, the rates obtained at pH 7.0 from different suspensions were used as the bases for adjusting the observed rates to what they would have been if a single suspension of constant activity could have been used. The

initial values of  $Q_{O_2}^h$  plotted against pH are shown in Fig. 9, the maximum being near pH 7.0.

To determine whether different rates of photooxidation (6) at different pH values might have been responsible for the lower rates of

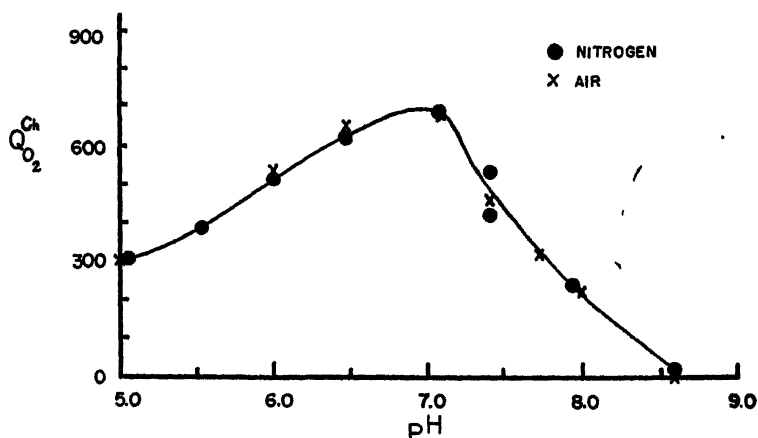


FIG. 9

The Rate of Oxygen Production at Various pH Values with Both Air and Nitrogen Atmospheres at 15°C., 0.28 mg. of Chlorophyll per Vessel

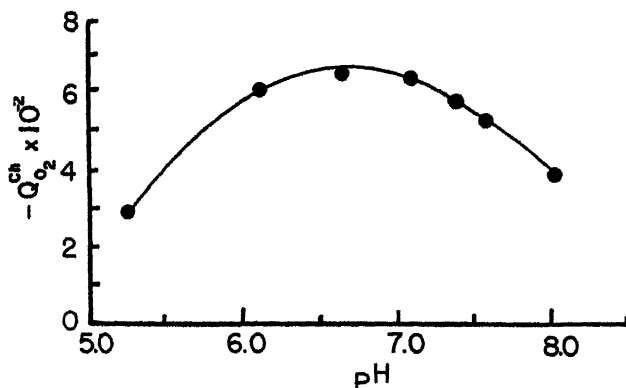


FIG. 10

The Effect of pH on the Rate of Photochemical Oxygen Uptake from an Air Atmosphere by Chloroplasts Immersed in Hill's Solution with Ferrieyanide Omitted 15°C., 0.23 mg. of chlorophyll per vessel

oxygen evolution found at the higher pH values, the rates of oxygen uptake when ferricyanide was omitted from Hill's solution were determined. The rates of photooxidation plotted as  $-Q_{O_2}^{ch}$  values against pH are given in Fig. 10. The maximum was near pH 6.8, showing that the lower rates of oxygen evolution at pH values other than pH 7.0 were not due to higher rates of photooxidation. This was further confirmed by the fact that the shape of the curve in Fig. 9, which was obtained for oxygen evolution with a nitrogen atmosphere, was identical with that obtained with an air atmosphere.

### 5. The Effect of Ferricyanide Concentration

The rates of oxygen evolution for five different ferricyanide concentrations at pH 6.9 were determined by the manometric method. The results are given in Table II. Apparently the optimum concentration is near 0.02 M, although the greatest variation from the maximum rate for the other concentrations is but 10%.

TABLE II

*The Rate of O<sub>2</sub> Evolution by Chloroplasts in Hill's Solution  
Containing Different Concentrations of K<sub>3</sub>Fe(CN)<sub>6</sub>*

Ferricyanide concentration	Rate of reaction
moles/liter	$Q_{O_2}^{ch}$
0.005	610
0.010	626
0.020	676
0.040	641
0.060	611

### 6. The Effect of Chloroplast Concentration

In order to determine whether the rate of the Hill reaction was directly proportional to the amount of chloroplast suspension present, the rates for various concentrations were determined by the manometric and the titration methods.

(a) *Titration Measurements:* With the light intensity used for this method (4500 foot candles of white light in front of the red filter) the rate of  $H^+$  production was directly proportional to the amount of chloroplast suspension present in the 50 ml. beaker up to and including the amount equivalent to 0.8 mg. of chlorophyll.

(b) *Manometric Measurements:* The aliquots of suspension used were equivalent to 0.095, 0.189, 0.284 and 0.378 mg. of chlorophyll.

The rates of oxygen evolution plotted against the respective amounts of chlorophyll are given in Fig. 11, and show that, for the light intensity used with this method (250 foot candles), the rates were not directly proportional to the amount of chloroplast suspension present over the practicable range of chlorophyll concentrations.

### 7. The Effects of Omitting Various Constituents of Hill's Solution

To determine which of the various constituents of Hill's solution would most limit the rate of oxygen evolution from spinach chloroplasts, various combinations were tried. The critical compound necessary for any oxygen evolution was found to be potassium ferricyanide.

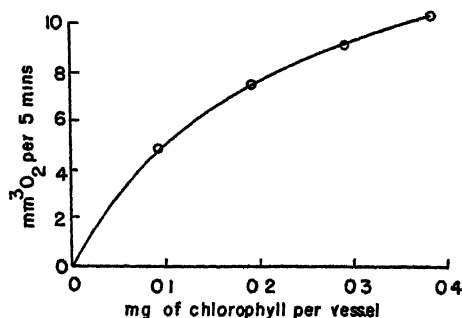


FIG. 11

The Effect of Chloroplast Concentration on the Rate of Oxygen Production  
15°C., pH 7.0

The concentrations of the compounds were kept the same as in complete Hill's solution.

In Fig. 12 are given the curves showing oxygen evolution from the following combinations: (1) complete Hill's solution, (2)  $K_2C_2O_4 + K_3Fe(CN)_6$ , (3)  $Fe(NH_4)(SO_4)_2 + K_3Fe(CN)_6$ , (4)  $K_3Fe(CN)_6$ , all at pH 6.8 with 0.017 M sodium sorbitol borate.

In order to compare accurately the rates of oxygen evolution from the above combinations, the pH at which the maximum rate of oxygen evolution occurs should be found in each case. This has been determined only in the case of  $K_2C_2O_4 + K_3Fe(CN)_6$  with both sodium sorbitol borate and phosphate buffers at 15°C. The curves in each case were almost identical. The data for the sodium sorbitol borate buffer is given in Fig. 13. The maximum rate was found to be near

pH 7.7. The shape of the curve on the acid side of the optimum is similar to that for Hill's solution, and there is a similar rapid falling off in rate near pH 8.0. This might show that the decline of the rate in an alkaline solution of complete Hill's solution is not due to the precipitation or binding of  $\text{Fe}^{+++}$  from Hill's solution as  $\text{Fe}(\text{OH})_3$  since  $\text{Fe}^{+++}$  was omitted in this experiment. The  $Q_{\text{O}_2}^{\text{ch}}$  values obtained from complete Hill's solution at pH 7.0 and from  $\text{K}_2\text{C}_2\text{O}_4 + \text{K}_3\text{Fe}(\text{CN})_6$  at pH

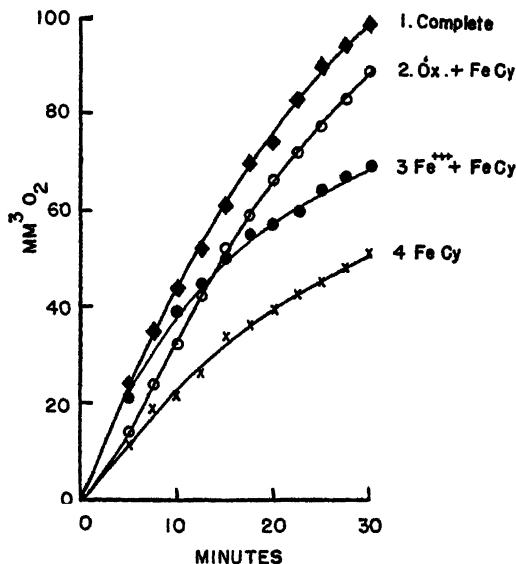


FIG. 12

Oxygen Production by Spinach Chloroplasts Immersed in Various Combinations of the Constituents of Hill's Solution

1.—Hill's solution; 2.—0.50  $M$   $\text{K}_2\text{C}_2\text{O}_4$  + 0.02  $M$   $\text{K}_3\text{Fe}(\text{CN})_6$ ; 3.—0.01  $M$   $\text{Fe}(\text{NH}_4)(\text{SO}_4)_2$  + 0.02  $M$   $\text{K}_3\text{Fe}(\text{CN})_6$ ; 4.—0.02  $M$   $\text{K}_3\text{Fe}(\text{CN})_6$ . 15°C., pH 6.8, all with 0.17  $N$  sodium sorbitol borate buffer. 0.28 mg. of chlorophyll per vessel.

7.7 were 436 and 403 respectively with the same chloroplast preparation, showing that the absence of  $\text{Fe}^{+++}$  from the reaction solution had little, if any, effect on the maximum rate attainable although it does affect the optimum pH value.

With potassium ferricyanide alone there was oxygen evolution, which was proved by the fact that, with 10% potassium hydroxide in one sidearm, yellow phosphorus in the inset, and a nitrogen atmos-

phere, no increase of gas volume occurred, while the identical components in another vessel without phosphorus gave a  $Q_{O_2}^{Ch}$  of 180.

### DISCUSSION

The possible connection between the Hill reaction and the  $O_2$  production step of photosynthesis has been discussed by Hill (2), Hill and Scarisbrick (4), French and Anson (6), and by French and Rabi-deau (8), and will not be dealt with here. The main point of this paper is to present the optimum conditions to be used in determining the rate of the Hill reaction and to show that, when ferricyanide is used as the oxidant for potassium ferrous oxalate, the equivalence of  $4Fe^{+++}$ ,

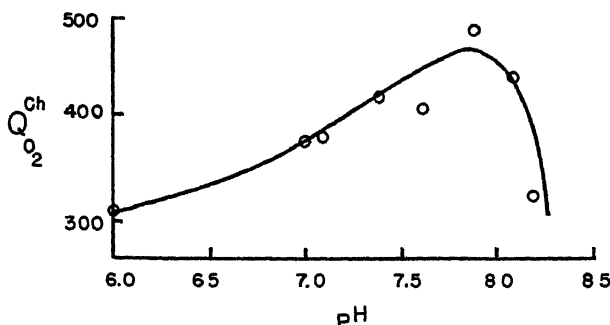


FIG. 13

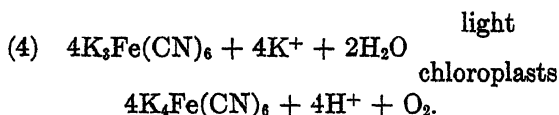
The Effect of pH on Oxygen Evolution by Spinach Chloroplasts Immersed in a Solution Containing  $0.50 M K_2C_2O_4 + 0.02 M K_3Fe(CN)_6$   
 $15^\circ C.$ , 0.26 mg. of chlorophyll per vessel

$4H^+$ , and  $O_2$  will be observed if certain conditions are used. The fact that  $H^+$  is produced enables one to measure the rate by means of titration, which is a much less time-consuming procedure than manometric measurement of oxygen evolution, and at the same time eliminates any possible specific buffer effects.

The fact that Hill and Scarisbrick (4) used their hemoglobin and methemoglobin methods to determine the effect of pH and of temperature on the rate of the reaction makes direct comparison of their results with ours impossible. They report that the values they found were concerned chiefly with the effect of pH and temperature on the reagents rather than on the active components of the chloroplasts. A possibly significant finding presented here is the fact that when illuminated



spinach chloroplasts are immersed in a solution containing no potassium ferric oxalate, but containing only ferricyanide, sodium sorbitol borate buffer and sucrose, oxygen is evolved. Hill and Scarisbrick (5) report that no oxygen was evolved from illuminated suspensions of *Stellaria media* and *Chenopodium Bonus Henricus*, immersed in potassium ferricyanide alone. One explanation for this seemingly anomalous  $O_2$  production may depend on the possibility of the natural occurrence of potassium ferric oxalate within spinach chloroplasts. Kohman (9) found the oxalate concentration in spinach leaves equivalent to about 9.0% anhydrous oxalic acid on a dry weight basis. Also, Liebich (10) reported 0.05% Fe in spinach chloroplasts on a dry weight basis. No information has been obtained as yet concerning oxalate concentration in the chloroplasts. Another explanation could be the replacement of ferric oxalate by ferricyanide. Hill (2) hypothesized that a readily oxidized and reduced substance A within chloroplasts is reduced when the chloroplasts are illuminated, and that the ferric oxalate complex reoxidized the reduced form of A. If ferricyanide does react with the reduced form of A, the reaction for the reduction of ferricyanide would probably be:



Before this can be confirmed, further study must be made with suspensions of chloroplasts prepared from plants known to have a negligible oxalate content.

#### SUMMARY

1. The rate of the Hill reaction, which results in the simultaneous production of  $O_2$  and  $H^+$  by illuminated chloroplasts, can be measured by constant pH titration.

2. For every four moles of ferricyanide reduced one mole of  $O_2$  and four moles of  $H^+$  are produced.

3. Photodecomposition of Hill's solution, which results in  $H^+$  production, is eliminated when a red filter removing wave lengths below 568  $m\mu$ . is used. Photooxidation, which occurs simultaneously with the Hill reaction, is reduced to a minimum by using a nitrogen atmosphere.

4. The temperature coefficient of the Hill reaction is about 3.5 for the interval 3–15°C., while that of the inactivation of chloroplasts in Hill's solution is about 3.9 over the same interval.

5. At 15°C. the maximum rate of oxygen evolution measured manometrically was at pH 7.0. With the titration method the maximum rate of  $H^+$  production was at pH 7.0 at 10°C., and at pH 7.6 at 3°C.

6. Photooxidation by chloroplasts suspended in a solution containing potassium ferric oxalate, sodium sorbitol borate and sucrose was found to have a pH maximum near pH 6.8 at 15°C.

7. The rate of oxygen evolution is essentially independent of the ferricyanide concentration between 0.005 and 0.06 *M* at 15°C.

8. Spinach chloroplasts suspended in a solution containing only potassium ferricyanide, sodium sorbitol borate and sucrose, when illuminated, evolve  $O_2$  at about one-half of the rate obtained with the complete Hill's solution.

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# The Urinary Excretion of Trigonelline- and Nicotinic Acid-Like Substances in Human Subjects After the Ingestion of Trigonelline and After Smoking <sup>1</sup>

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## INTRODUCTION

Patients with nicotinic acid deficiency consistently excrete less "trigonelline" than normally nourished individuals. The urinary excretion of "trigonelline" was, therefore, proposed as a measure of nicotinic acid nutrition (1, 2). More recently it was found that a large portion of the metabolite appearing in the urine after the ingestion of nicotinic acid is not trigonelline, but its amide, or N-methylnicotinamide (3, 4, 5, 6). Since the analytical method used by us does not differentiate between trigonelline and N-methylnicotinamide all our values will be given in terms of "trigonelline." The term trigonelline will be reserved to the pure crystalline substance administered orally. Many components of man's diet (coffee, tea, chocolate, legumes and nuts) contain large amounts of trigonelline which is excreted in the urine. Unless these foods are avoided, the urinary "trigonelline" is not a true index of nicotinic acid metabolism (7). Likewise, the nicotine in tobacco is partially absorbed and excreted in the urine, thus increasing the apparent nicotinic acid value (8, 9). Abstinence from tobacco and coffee is difficult to obtain even under hospital surveillance for short periods of time.

The purpose of this study was to determine the length of time required for the complete excretion of absorbed nicotine and ingested trigonelline, in patients on a constant diet.

<sup>1</sup>The expenses of this investigation were defrayed by the Upjohn Company, Kalamazoo, Mich. and the Horace H. Rackham School of Graduate Studies, University of Michigan, Ann Arbor, Mich.

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Preliminary determination showed that the excretion of "trigonelline" after ingestion of coffee and other substances is not constant, due probably to variations in trigonelline content. In the case of coffee, great variations were found depending on the brand and the method of brewing. A standard oral dose of trigonelline was therefore used.

### EXPERIMENTAL

1. *The Urinary Excretion of Ingested Trigonelline.* "Trigonelline" was determined by the method of Fox, McNeil and Field (10).

Seven patients with peptic ulcer were used in this study. They were on a Sippy diet, which is constant and very low in trigonelline. During the experimental period

TABLE I

*Excretion of Trigonelline-Like Substances After Oral Administration of a 200 mg. Test Dose of Trigonelline Sulfate*

All values expressed as mg./4 hr.

		Subjects							
Hours		J.P.	C.M.	S.J.	P.L.	L.F.	E.R.	J.S.	Av.
Before Test Dose		5.0	4.0	2.4	2.7	4.7	4.0	1.3	} 3.3
		4.8	4.0	2.4	1.5	3.0	4.0	1.5	
		4.8	—	—	—	—	—	—	
		4.6	—	—	—	—	—	—	
		5.0	—	—	—	—	—	—	
After Test Dose	4	23.0	17.6	37.7	9.5	14.7	2.9	9.8	16.4
	8	79.0	50.1	26.5	32.9	42.9	2.2	36.7	38.6
	12	52.0	13.3	16.0	23.3	44.7	17.5	31.8	31.2
	16	58.0	17.7	14.5	11.6	9.5	12.0	—	20.5
	20	—	—	—	—	—	—	27.8	—
	24	46.0	15.0	—	8.4	9.0	13.3	10.5	17.0
	28	4.6	9.7	10.1	14.4	13.3	5.2	7.4	9.2
	48	1.2	3.8	2.2	0.4	9.3	1.5	6.3	3.5
	72	0.5	—	1.5	0.3	1.6	1.2	—	1.0
	96	—	—	—	—	—	—	0.9	—

the patients received no other food than 2 ounces of cream every 2 hours. After at least two 24 hour basal determinations of urinary "trigonelline," 200 mg. of trigonelline sulfate in a gelatin capsule were fed. The urine was then collected at 4 hour intervals for 16 hours and then after 24, 48 and 72 hours.

The urinary "trigonelline" rose sharply from an average basal value of 3.3 mg./4

hours to an average value of 38.6 mg./4 hours. The peak was reached in 4 to 12 hours, after which the excretion of trigonelline-like substances gradually decreased and returned to normal or sub-normal values 48 to 72 hours after the ingestion of the test dose.<sup>3</sup> (See Table I.)

It is apparent that the excretion of ingested trigonelline is a relatively slow process and that at least two days should elapse between the last ingestion of coffee or other trigonelline-containing foods and the determination of urinary "trigonelline" for the purpose of examining the status of nicotinic acid nutrition.

TABLE II

*Effect of Smoking on the Urinary Excretion of Nicotinic Acid- and Trigonelline-Like Substances*

All values expressed in milligrams of nicotinic acid and trigonelline per 24 hours

Patient	Basal 3 day av.		Smoking 7 to 25 cigarettes		Day following smoking	
	"Nicotinic acid"	"Trigonelline"	"Nicotinic acid"	"Trigonelline"	"Nicotinic acid"	"Trigonelline"
B.K.	11.6	38.7	13.0	24.0	10.0	29.0
P.F.	8.3	35.7	10.0	29.0	—	—
S.L.	8.0	18.7	9.5	13.0	9.0	9.7
B.M.	11.0	22.7	16.0	14.0	10.0	13.2
O.K.	12.6	17.3	18.0	17.0	14.0	13.0
E.M.	9.0	21.7	13.0	12.0	10.0	15.0
R.F.	—	18.3	—	12.7	—	10.0
S.F.	—	29.7	—	28.0	—	20.0
P.H.	—	14.7	—	26.7	—	15.0
Average	10.1	23.7	13.4	19.6	10.6	13.7

2. *The Effect of Tobacco on the Excretion of Nicotinic Acid- and Trigonelline-Like Substances.* Twelve experiments were performed on 9 normal laboratory workers. In 9 cases no attempts were made to control the diet other than the elimination of coffee, tea, chocolate, beans and nuts. Three basal 24 hour collections of urine were made. The subjects then smoked 7 to 15 cigarettes in one day making an effort to inhale the smoke. The urine specimens excreted during the smoking day and the following 24 hour period were collected separately.

"Nicotinic acid" was determined by the method of Melnick and Field (11).<sup>4</sup> The

<sup>3</sup> The administration of trigonelline was followed by an increase in the excretion of nicotinic acid-like substances, an unexpected result which will be discussed in a separate note as it has interesting implications.

<sup>4</sup> The method is not specific for nicotinic acid but determines many other substances containing the pyridine ring, provided the nitrogen is trivalent. It will determine, therefore, nicotinamide, nicotinuric acid and nicotine, but not trigonelline and N-methyl-nicotinamide (11).

average 24 hour excretion of "nicotinic acid" was 10.1 mg. It rose to 13.4 mg. during the smoking period and returned to 10.6 mg. 24 hours later. (See Table II.) The results were qualitatively the same in all experiments, although the increase in nicotinic acid-like substances was greater when the smoke was more deeply inhaled. Only in one experiment (P.H.) did the excretion of "trigonelline" increase during the smoking period.

In 3 experiments the subjects ate only milk; the urine was collected every 2 hours for 8 to 16 hours. Each period of smoking was followed by two periods in which the subject did not smoke. In one case, a final sample of urine was collected 24 hours after the beginning of the experiment. (See Table III.)

TABLE III

*Effect of Tobacco on the Urinary Excretion of Trigonelline- and Nicotinic Acid-Like Substances*

All values expressed in mg. of trigonelline and nicotinic acid per 2 hours

Hours	B.M.			N.F.			P.F.		
	Cigar-ettes	"Trigo-nelline"	"Nico-tinic acid"	Cigar-ettes	"Trigo-nelline"	"Nico-tinic acid"	Cigar-ettes	"Trigo-nelline"	"Nico-tinic acid"
0-2	0	6.3	0.9	0	5.7	2.2	0	15.0	1.7
2-4	0	3.8	0.9	0	4.0	2.7	0	7.5	1.6
4-6	9	3.4	3.8	4	4.1	4.7	5	5.0	2.2
6-8	0	2.8	2.4	0	2.8	4.0	0	5.2	3.9
8-10	0	2.4	1.9	0	4.4	2.5	0	4.0	1.4
10-12	7	2.4	3.2	—	—	—	5	3.6	1.8
12-14	0	3.7	1.2	—	—	—	Dinner	3.2	2.7
14-16	—	—	—	—	—	—	0	4.7	1.6
16-18	—	—	—	—	—	—	3 Pipes	8.6	1.6
18-20	—	—	—	—	—	—	0	6.0	3.0

A rise in the apparent urinary excretion of "nicotinic acid" occurs during each smoking period and is sometimes still apparent in the following 2 hour period. The excretion of "trigonelline" was not affected by smoking.

The gradual decline of "trigonelline" excretion during the experiment was probably due to the slow elimination of the trigonelline ingested with food in the days preceding the experiment. In contrast to this the excretion of nicotine seems to be rapid.

When the excretion of nicotinic acid-like substances is used for the diagnosis of subclinical deficiency, smoking should be avoided at least during the period in which urine is collected. Smoking is permissible if "trigonelline" excretion is used as an index of nicotinic acid nutrition.

## SUMMARY

1. Following the oral administration of 200 mg. of trigonelline, the urinary excretion of trigonelline-like substance is increased and returns to normal after 48 to 72 hours. Coffee, chocolate, tea and legumes, which contain relatively large amounts of trigonelline, should be eliminated from the diet for at least 2 days preceding the chemical determination of "trigonelline" in the urine.

2. Smoking is followed by an apparent increase in nicotinic acid excretion, probably due to the presence of nicotine in the urine. Smoking should be prohibited during the collection of urine for the determination of "nicotinic acid."

3. Smoking does not increase the apparent urinary excretion of "trigonelline."

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# Studies on the Biochemistry of *Tetrahymena*

## VI. Folic Acid as a Growth Factor for *T. Geleii* W

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### INTRODUCTION

Until the discovery that two of the "unknown growth factors" for *Tetrahymena geleii* W could be replaced with chemically identified substances (1) and that Factor II was not readily adsorbed on activated charcoal, it had been impossible to determine whether or not folic acid functioned as a growth requirement for this ciliate. When it was necessary to supply Factor I as the lead acetate precipitate fraction of crude extracts, and this factor was found to be readily adsorbed on activated charcoal it was apparent that any folic acid in the extracts would always be found in this fraction. With the substitution of purines (guanine, adenylic acid) for Factor I and pyrimidines (cytidylic acid, uridylic acid) for Factor III the role of folic acid could be determined. Folic acid has been found to be a growth requirement for *T. geleii* W and the quantitative data presented below are important when it is noted that this is the first organism of animal nature to be studied under aseptic conditions in relation to this growth substance

### EXPERIMENTAL

The organism used in this study was *Tetrahymena geleii* W, the strain which was used in earlier investigations (1, 2, 3, 4, 5) grown in pure (bacteria-free) culture. The base medium used is given in Table I.

The base medium was tested for folic acid with *Lactobacillus casei* using the amino acid base medium of Hutchings and Peterson (6). This was especially important for the Factor II preparation as all the other constituents were of chemically known constitution. The Factor II preparation used was the same as that employed in the study of nucleic acid and its derivatives (1) and it was found that after the Norit treatment all of the folic acid had been removed from the lead acetate filtrate fraction

of Liver fraction L<sup>1</sup>. When the Factor II preparation was added to the *L. casei* base medium in concentrations up to four times that used for the ciliate no growth of the bacteria occurred in the absence of added folic acid. The addition of folic acid, however, resulted in optimum growth of the bacteria showing that these concentrations of Factor II are not toxic.

Although yeast nucleic acid (hydrolyzed) had previously been found to permit somewhat better growth of *Tetrahymena* than the purines and pyrimidines used (1) it was entirely unsatisfactory for this work as it contains appreciable amounts of folic acid.

TABLE I  
Final Concentrations of Constituents of Base Medium

	mg./ml.		γ/ml.
<i>l</i> -(+)-Arginine monohydrochloride.....	0.82	Biotin methyl ester.....	0.00005
<i>l</i> -(-)-Histidine monohydrochloride.....	0.10	Calcium pantothenate . . . . .	0.10
<i>dl</i> -Isoleucine.....	0.35	Thiamin hydrochloride.....	0.10
<i>dl</i> -Leucine.....	0.35	Nicotinamide.....	0.10
<i>dl</i> -Lysine.....	0.60	<i>p</i> -Aminobenzoic acid.....	0.10
<i>dl</i> -Methionine . . . . .	0.34	Pyridoxine hydrochloride....	0.10
<i>dl</i> -Phenylalanine . . . . .	0.14	Riboflavin . . . . .	0.10
<i>dl</i> -Serine . . . . .	0.04	<i>l</i> -Inositol . . . . .	1.00
<i>dl</i> -Threonine . . . . .	0.20	Choline chloride.....	1.00
<i>l</i> -(-)-Tryptophane.....	0.10		mg./ml.
<i>dl</i> -Valine.....	0.20	Guanine hydrochloride.....	0.05
Dextrose.....	2.00	Adenylic acid.....	0.05
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.10	Cytidylic acid . . . . .	0.05
K <sub>2</sub> HPO <sub>4</sub> .....	0.10	Uridylic acid.....	0.05
CaCl <sub>2</sub> ·2H <sub>2</sub> O.....	0.05		
FeCl <sub>3</sub> ·6H <sub>2</sub> O . . . . .	0.00125	Factor II preparation added in concentrations of 1 part in 10 parts of final medium	
MnCl <sub>2</sub> ·4H <sub>2</sub> O . . . . .	0.00005		
ZnCl <sub>2</sub> .....	0.00005		

All experimental series were carried out in 2 ml. volumes of medium in 125 × 7 mm. Pyrex test tubes plugged with Pyrex wool (5). In all cases three serial transplants were made with bacteriological loops and the population densities were determined in the third serial transplant after 72 hours of incubation at 25°C. All glassware was made chemically clean with dichromate-sulfuric cleaning solution followed by thorough rinsing and air-drying. All media were made with water twice distilled over permanganate in an all-Pyrex still, and sterilization was by autoclaving.

When the base medium, without folic acid, was inoculated with *Tetrahymena geleii* W from a heavy culture, growth occurred in the

<sup>1</sup> Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

first transplant due to the carry-over of folic acid both in the medium and in the ciliates. That the ciliates, grown in the presence of folic acid, store appreciable amounts of the growth factor was demonstrated by washing the organisms aseptically by centrifugation. After four washes in folic acid-free medium (10 ml. at each change) the inoculated ciliates grew to moderate concentrations in the first transplant in the base medium. The second transplant, however, was very low and the third invariably negative.

The addition of folic acid <sup>2</sup> (0.1 $\gamma$  per ml.) to the base medium resulted in optimum and indefinitely transplantable growth, under these conditions, and demonstrated that this growth factor was essential for this organism.

In a scanning test for the effective concentrations of folic acid for *T. geleii* W the growth factor was added in serial dilutions from 10 $\gamma$  per ml. to 0.000001 $\gamma$  per ml. From the results of this test it was possible to determine the effects of graded doses of folic acid in the effective range (0.001 $\gamma$  per ml. to 0.05 $\gamma$  per ml.). This experiment was repeated three times and the figures averaged and plotted. These data are presented in Fig. 1.

With a folic acid concentrate (potency of 5000) dosage range from 0 to 0.02 $\gamma$  per ml. a linear response occurs. Above this concentration smaller population increments result until, at 1.0 $\gamma$  per ml., the population produced has reached approximately 200,000 ciliates per ml. This figure represents the maximum under these conditions and the addition of up to 10.0 $\gamma$  per ml. of folic acid causes no significant increase. Calculated on the basis of potency 40,000 (7, 8, 9) it is seen that the amount of folic acid required for 1/2 maximum growth of *T. geleii* W is 0.002 $\gamma$  per ml.

The amount of folic acid necessary for optimum growth is much greater than previously supposed. In earlier investigations on *Tetrahymena* (2, 3, 5) only 0.001 $\gamma$  per ml. of folic acid concentrate was added to the base medium. The "unknown growth factor" preparations (Factors I, II and III) contained adequate amounts of folic acid, however. When purines and pyrimidines were substituted for Factors I and III, and the Factor II was supplied as the Norit treated lead acetate filtrate of Liver Fraction L, the amount of folic acid concentrate

<sup>2</sup> The folic acid used was in the form of a concentrate with a potency of 5000 furnished through the courtesy of Dr. R. J. Williams.

added to the base media was arbitrarily increased to 0.1  $\gamma$  per ml. (1). It now is apparent that even this increase was insufficient for maximum growth. It also explains the difference in population yield between media containing hydrolyzed nucleic acid and mixtures of purines and pyrimidines, for the nucleic acid, as noted earlier (1), contains folic acid.

Inasmuch as *Tetrahymena* is distinctly animal-like in its amino acid requirements (2, 4) it appears that the data presented here are of

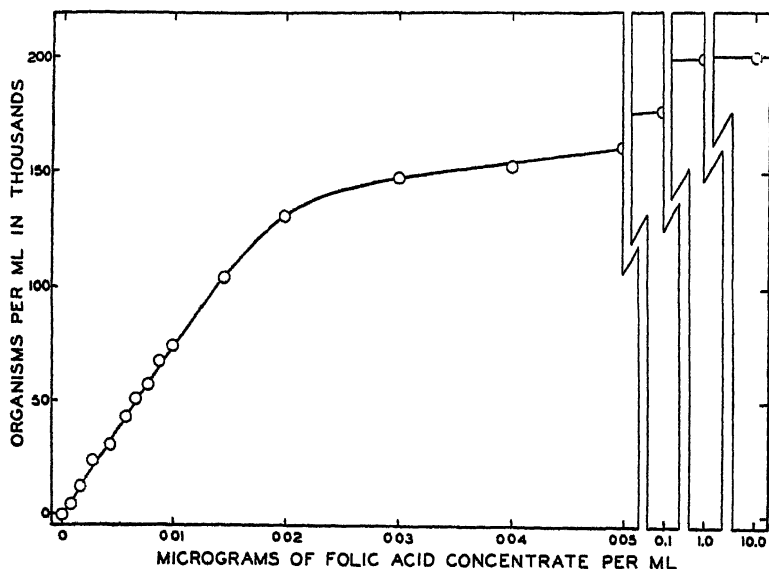


FIG. 1

Dosage-response Curve Showing the Population of *T. geleii* W in Relation to the Concentration of Folic Acid Concentrate (potency 5000)  
Each point represents an average of four separate experiments. The populations are from the third serial transplant incubated 72 hours

somewhat more importance than merely to show that still another organism requires folic acid. They indicate the relatively high requirement of at least one animal and may prove useful in future investigations with other species. *Tetrahymena* should be a valuable organism in testing the similarities and differences in physiological function between folic acid and other closely related substances (Vitamin B<sub>9</sub>, etc.).

## SUMMARY

The ciliate *Tetrahymena geleii* W requires folic acid in relatively high concentrations. This growth factor allows population increases in direct proportion to the amount added through the effective range. This is the first animal grown under aseptic conditions for which quantitative data are available for this growth factor.

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# Reagents for the Detection of Hydrogen Peroxide Production by Bacteria in Plate Cultures (with Notes for the Preparation of 2,7-Diaminofluorene Dihydrochloride)<sup>1</sup>

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## INTRODUCTION

Penfold (14), noting the black pigment produced by pneumococci and streptococci on unheated blood agar containing benzidine, suggested the differential value of such a medium. The pigment was believed to be an oxidation product and benzidine was superior for the reaction to *p*-phenylenediamine,  $\beta$ -naphthylamine, pentamethylenediamine hydrochloride or tyrosin.

Additional evidence appeared correlating the blackening of blood-benzidine media with hydrogen peroxide production (8, 9, 10). New interest in the test followed the report (6) that the reaction was produced by *Clostridium novyi* (*C. oedematiens*), an important agent of gas gangrene. Of the anaerobic organisms, only this type, *C. botulinum* and certain streptococci showed similar reactions. All strains of *C. novyi* investigated gave the characteristic blackening and the test was suggested as an identification reaction for this species in the study of the anaerobic flora of war wounds. Hayward (5) indicated that the blackening of heated blood might be due to proteolytic action and pointed out that darkening of benzidine agar should be used as an indicator of hydrogen peroxide only if unheated blood is used. Evidence was also presented that the reaction was not specific for *C. novyi* since some strains of this species failed to produce peroxide in quantities sufficient to give a recognizable reaction, and, further, *C. multifementans*, *C. cochlearium* and *C. botulinum* were positive. The value of this reaction as a specific aid for the identification of *C. novyi* may be diminished in view of the above and it may also be supplanted by another test proposed by Nagler (13), which does not depend upon peroxide production. We had investigated, prior to the appearance of Nagler's article, other reagents for

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<sup>1</sup> The authors wish to acknowledge the technical assistance of Ruth Toabe and Andrew Albright, PhM 1/C, USN.



the blackening reaction. Our interest in these compounds stemmed from the possibility that another substance might be discovered which would be more sensitive than benzidine when used with unheated blood (rabbit) agar. Since the results may be of some interest in the general reaction, they are summarized below.

## EXPERIMENTAL

### *Reagents*

The following compounds were tested: (1) 2,4-diaminophenol dihydrochloride, (2) *o*-phenylenediamine dihydrochloride, (3)  $\alpha$ -naphthylamine hydrochloride, (4)  $\beta$ -naphthylamine hydrochloride, (5) phenylhydrazine, (6) *p*-aminobenzophenone, (7) 4,4'-diaminodiphenylmethane dihydrochloride, (8) 4,4'-diaminodiphenylether dihydrochloride, (9) diphenylamine, (10) 5-chloro-2-aminotoluene hydrochloride, (11) diethylamine hydrochloride, (12) *p*-aminodiethylaniline monohydrochloride, (13) *o*-aminodiphenyl hydrochloride, (14) hydroquinone, (15) dimethyldihydroresorcinol, (16) 1,8-diaminonaphthalene-3,6-disulfonic acid, (17) methylamine hydrochloride, (18) di-*n*-butylaniline hydrochloride, (19) di-*n*-butylamine hydrobromide, (20) ethyl *p*-aminobenzoate hydrochloride, (21) 2,7-diaminofluorene dihydrochloride, (22) benzidine dihydrochloride.

The benzidine dihydrochloride used was c.p. grade (Coleman and Bell Co.) and all other compounds, except the 2,7-diaminofluorene dihydrochloride, were prepared by commonly available methods. The 2,7-diaminofluorene dihydrochloride has been prepared previously by the reduction of 2,7-dinitrofluorene with tin and hydrochloric acid (11, 12, 16, 17), by the use of ammonium polysulfide (1) or sodium hydrosulfite (11), and by the action of tin and hydrochloric acid on 2-amino-7-nitrofluorene (2). In most instances, these methods give a poor yield. We have found that 2,7-dinitrofluorene may be reduced to 2,7-diaminofluorene dihydrochloride in a 96% yield by catalytic reduction in the presence of hydrogen, absolute alcohol, and platinum oxide, followed by treatment with dry hydrogen chloride.

*2,7-Dinitrofluorene.* This compound was prepared by nitration of fluorene with fuming nitric acid and glacial acetic by the method of Morgan and Thomason (11) (m.p. 269°C. with decomposition).

*2,7-Diaminofluorene dihydrochloride.* Suspend 5.12 g. (0.02 mole) of 2,7-dinitrofluorene in 200 ml. of absolute alcohol in the reduction bottle of a low pressure catalytic hydrogenation apparatus, and add 0.1 g. of platinum oxide catalyst. Evacuate apparatus, then fill with hydrogen and heat the mixture to 60°C. in the reduction bottle which has previously been wound with resistance wire and taped to prevent slippage of the coil. The coil is calibrated with water in the bottle, using a Variac transformer. Simultaneously with the heating, shake mixture with hydrogen, at an initial pressure of 45 pounds per square inch, until twelve hundredths of a mole are absorbed (approximately forty-five minutes). Leave bottle in the apparatus and cool to room temperature. Add 15 ml. of petroleum ether (30–60°) and filter the solution into a wide mouth 500 ml. Erlenmeyer flask, which contains 25 ml. of just boiling petroleum ether (30–60°). If the 2,7-diaminofluorene crystallizes on the filter paper, wash through with 10 ml. of absolute alcohol followed by 10 ml. of petroleum ether (30–60°). *Immediately* saturate the filtrate, which is reddish-yellow in color,

with dry hydrogen chloride gas until precipitation of the diamine is complete. Filter the 2,7-diaminofluorene dihydrochloride rapidly, washing with two 25 ml. portions of 30–60° petroleum ether, and dry as quickly as possible, as the diamine or its salts darken quickly if wet. The yield is 4.59 g. (85.8% of theoretical) of cream colored powder. If the filtrate is evaporated *in vacuo* to a volume of 50 ml. and 50 ml. of peroxide free, dry ether is added, an additional 0.55 gm. of the product may be

TABLE I

*Color Changes Produced on Mixing Solutions of Various Compounds with Hydrogen Peroxide and Horse Radish*<sup>1</sup>

Number	Compound <sup>11</sup> Name	Reaction Horse radish plus H <sub>2</sub> O <sub>2</sub>
1	2,4-Diaminophenol dihydrochloride	blackish purple → dark maroon purple
2	<i>o</i> -Phenylenediamine dihydrochloride	xanthine orange
3	$\alpha$ -Naphthylamine hydrochloride	deep soft blue violet
4	$\beta$ -Naphthylamine hydrochloride	mauve
5	Phenylhydrazine hydrochloride	negative
6	<i>p</i> -Aminobenzophenone	negative
7	4,4'-Diaminodiphenylmethane dihydrochloride	salmon
8	4,4'-Diaminodiphenylether dihydrochloride	flesh
9	Diphenylamine	light mauve
10	5-Chloro-2-aminotoluene hydrochloride	dark anthracene violet
11	Diethylamine hydrochloride	negative
12	<i>p</i> -Aminodiethylaniline monohydrochloride	dahlia carmine
13	<i>o</i> -Aminodiphenyl hydrochloride	grayish blue violet
14	Hydroquinone	buff yellow
15	Dimethyldihydroresorcinol	negative
16	1,8-Diaminonaphthalene-3,6-disulfonic acid	pale green
17	Methylamine hydrochloride	negative
18	<i>n</i> -Dibutylaniline hydrochloride	sulphate green
19	Di- <i>n</i> -butylamine hydrobromide	negative
20	Ethyl <i>p</i> -aminobenzoate hydrochloride	negative
21	2,7-Diaminofluorene dihydrochloride	indulin blue
22	Benzidine dihydrochloride	dusky blue violet

\* Freshly cut, unheated cabbage tissue gave similar color changes. Heating the tissue (boiling water for 10 minutes) destroyed the activity. All color changes are recorded according to the system of Ridgway (15).

\*\* Concentration: 0.5%.

obtained. The total yield is 96% of the theoretical amount. As a sample of the diamino dihydrochloride darkened slightly over a period of six months when stored at room temperature in a colorless glass bottle, it is best to keep the compound in a well stoppered brown bottle.

The color changes, recorded according to Ridgway's system (15), evidenced on mixing a water solution (suspension) of the various compounds with a solution of

hydrogen peroxide and peroxidase from cabbage and horse radish are shown in Table I. No color change appeared unless hydrogen peroxide was added.

### *Cultures and Culture Methods*

The bacteria tested included: three strains of *C. novyi* (*C. oedematiens*)<sup>2</sup> (Nos. 19, 112, 122), one strain each of *C. paratubulinum*<sup>3</sup> (No. 492), and *Streptococcus liquefaciens*, two strains of *Diplococcus pneumoniae*, and four other unidentified streptococci. These were streaked on plates (clay top dishes) of the following medium: Bactopeptone, 10 g.; glucose, 2 g.; NaCl, 1 g., liver extract, 100 ml.; distilled water, 900 ml.; and agar, 20 g.; pH 7.2. Defibrinated rabbit blood (unheated) was added to the sterile base medium, after cooling to 40°C., in the proportions of 1 ml. of blood to 9 ml. of medium. The pneumococci were also grown on a specially prepared beef heart base medium with blood (pH 7.6) as growth on the above medium was scanty and the reactions indefinite.

The compounds tested in this series were prepared in 0.15% and 0.5% concentrations by adding the desired amount, weighed on an analytical balance, to a measured volume of sterile tap water. The reagents were not heat sterilized or filtered. One ml. of the desired concentration was added to 9 ml. of the blood medium. All plates were incubated at 37°C. and observations were recorded of the aerobic series at 24, 48 and 72 hours. The clostridia were incubated in the vegetable tissue jar (7) and observed only at the end of 48 hours. The *p*-aminodiethylaniline dihydrochloride (No. 12) caused considerable darkening of the uninoculated medium.

### RESULTS

Of all the compounds tested only benzidine and 2,7-diaminofluorene dihydrochloride were suitable. With each of these reagents, positive cultures (*C. novyi*, *Diplococcus pneumoniae*, and two of the unidentified streptococci) developed a pigment (blue-black) which was easily recognizable by reflected light and was especially characteristic with transmitted light. Both of the concentrations gave clear results. In some instances, a similar reaction was also observed with the 0.5% concentration of *p*-aminodiethylaniline dihydrochloride but the general darkening of the medium which appeared even in the uninoculated control plates interfered with easy recognition.

<sup>2</sup>There is some confusion concerning the proper name for this species. For the purposes of this paper, *C. novyi* and *C. oedematiens* may be considered to be synonymous.

<sup>3</sup>This species is referred to as *C. botulinum* in the English literature (5, 6).

With the following reagents a dark brown pigmentation appeared with positive cultures which was more pronounced with transmitted light than with reflected light:  $\alpha$ -naphthylamine hydrochloride (0.5 and 0.15%),  $\beta$ -naphthylamine hydrochloride (0.15%), 4,4'-diaminodiphenylmethane dihydrochloride (0.15%), *p*-aminodiethylaniline monohydrochloride (0.15%), *o*-aminodiphenyl hydrochloride (0.15%), hydroquinone (0.15%), 1,8-diaminonaphthalene-3,6-disulfonic acid (0.15%), di-*n*-butylamine hydrobromide (0.5%). Dark colonies were produced with the following, but transmitted light was needed to distinguish positive from negative cultures: 2,4-diaminophenol dihydrochloride (0.5%), *o*-phenylenediamine dihydrochloride (0.5 and 0.15%), phenylhydrazine hydrochloride (0.15%), *p*-aminobenzophenone (0.15%), diphenylamine (0.15%), 5-chloro-2-aminotoluene hydrochloride (0.5 and 0.15%), diethylamine hydrochloride (0.15%), and methylamine hydrochloride (0.15%). In none of these did a positive reaction appear as definite as with benzidine or 2,7-diaminofluorene dihydrochloride. The reactions of the remaining compounds, or concentrations of others listed, were not sufficiently distinctive to be significant. 2,7-Diaminofluorene dihydrochloride has been proposed previously for detection of blood peroxidase in the presence of peroxide (16) and of peroxidase produced by bacteria (3, 4).

### SUMMARY

Of 22 organic compounds tested for usefulness in the demonstration of hydrogen peroxide production by bacteria on unheated rabbit blood agar, only benzidine dihydrochloride and 2,7-diaminofluorene dihydrochloride were satisfactory. With positive cultures, colonies appearing on streaked plates developed a blue-black pigmentation which was easily recognized by either reflected or transmitted light.

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# The Fermentation of Lactose by an Extract of *Streptococcus Thermophilus*<sup>1, 2</sup>

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## INTRODUCTION

It was shown by Wright (1) that certain strains of *S. thermophilus* fermented lactose and sucrose more rapidly than any other sugar. This was interpreted as evidence that these disaccharides were fermented directly, *i.e.*, without preliminary hydrolysis. Further experiments showed that when the fermentation of disaccharides was inhibited by iodoacetate and fluoride, no reducing monosaccharides accumulated, which again seemed to indicate that hydrolysis of disaccharides did not occur in these organisms. Later experiments, however, revealed that glucosides were not hydrolyzed in the presence of iodoacetate and it was concluded that the previous evidence was insufficient to preclude the hydrolysis of disaccharides before fermentation.

In the present study, a cell-free extract of *S. thermophilus* was prepared which fermented lactose and glucose, but not galactose, to lactic acid when adenosine triphosphate<sup>4</sup> was added. In addition, it was found that lactose was hydrolyzed rapidly by this extract.

## EXPERIMENTAL

*Culture of the organism.* The organism was maintained on skim milk. To obtain maximum yields of bacteria the following procedure was used. A medium containing 16 g. Difco broth, 10 g. lactose and 0.5 g. peptonized milk per liter was inoculated

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<sup>1</sup> This strain of *S. thermophilus* was obtained through the courtesy of Dr. J. M. Sherman, Cornell University.

<sup>2</sup> This work was supported by a grant from the Rockefeller Foundation.

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<sup>4</sup> Designated ATP in the rest of the paper.

and incubated at 48°C. until good growth was established. The medium was kept nearly neutral during the subsequent incubation by adding sterile sodium carbonate solution from a side arm of the incubation flask. After about fourteen hours the bacteria were centrifuged and washed with 0.9% sodium chloride. Such cultures yielded about 450 mg. dry bacteria per liter of medium.

*Preparation of the extract.* Washed fresh cultures were dried *in vacuo* over phosphorus pentoxide. The dried bacteria were ground with twice their weight of grade FFF carborundum powder in a cold mortar. Phosphate buffer, 0.02 *M*, pH 7.4 was added slowly during the grinding. After centrifugation, an opalescent solution was obtained. In the experiments cited, 1 ml. of extract represented 33 mg. of dry bacteria.

*The fermentation of sugars.* Extracts prepared as described above could ferment lactose and glucose only in the presence of ATP. The behavior of sugar-extract-ATP mixtures is shown in Fig. 1. All solutions were buffered to avoid initial pH changes.

It may be seen that no fermentation occurred in flask 1 until ATP was tipped in. Incubation for two hours in the absence of ATP apparently inactivated no enzymes, since the first part of curve 3 is nearly identical with curve 1 after addition of ATP. Curve 2 indicates acid production due to splitting of ATP by an adenyl pyrophosphatase present in the extracts.

The table accompanying Fig. 1 shows the amount of lactic acid present at the end of the experiment. Only in the flasks containing ATP and glucose or lactose was appreciable lactic acid produced.

Under the conditions of this experiment one acid equivalent is liberated and measured as carbon dioxide when a phosphate group is transferred from ATP to sugar, a reaction which is not inhibited by fluoride or iodoacetate. The initial sharp rise in carbon dioxide production in curves 3, 4, 6 and 7 is probably due to this reaction, while the flattening of curves 6 and 7 is due to the inhibition of fermentation by iodoacetate and fluoride, respectively. It may be seen that the curve for galactose (curve 5) is significantly lower than curves 6 and 7. This sugar apparently is neither phosphorylated nor fermented to any appreciable extent by this extract.

*The fermentation of phosphorylated sugars.* The results obtained are given in Table I. It is apparent that  $\alpha$ -glucose-1-phosphate is fermented rapidly. Neither  $\alpha$ -galactose-1-phosphate (2) nor  $\beta$ -galactose-1-phosphate (3) was fermented appreciably. Very little phosphatase active at pH 7.6 was present in the extracts, for phosphorus was not split from the above intermediates nor from  $\beta$ -glycerophosphate. Lactose could not be fermented as no ATP was added.

*The hydrolysis of lactose.* The data in Table II indicate that a lactase is present in extracts of dried *S. thermophilus* which hydrolyzes lactose rapidly. Whether this enzyme plays an important role in the metabolism of lactose by the live organism is a question to be settled by further investigation. Preliminary experiments indicated that at 42°C.

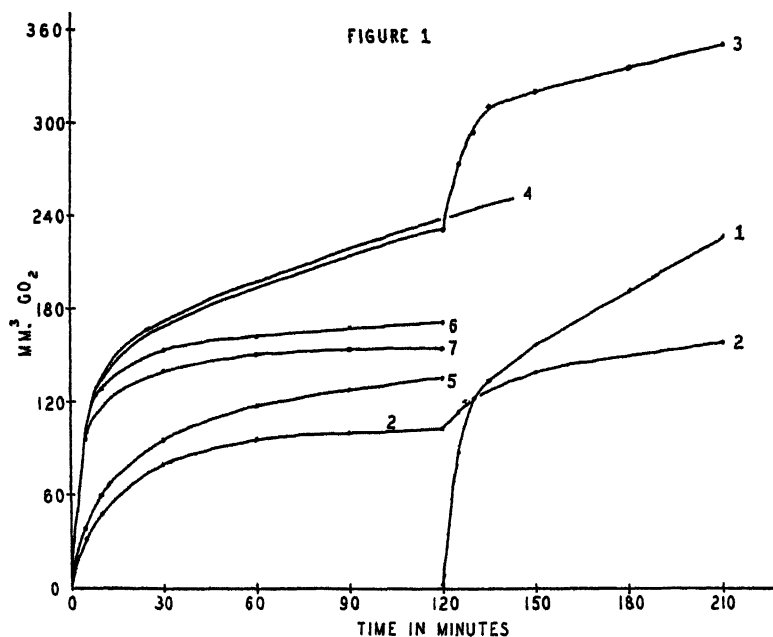


FIG. 1

Flask	Sugar	Sidearm 1 ATP	Inhibitor	Sidearm 2 ATP	Lactic Acid in $\gamma$
1	Lactose	—	—	+	74.9
2	—	+	—	+	13.2
3	Lactose	+	—	+	99.0
4	Glucose	+	—	—	79.0
5	Galactose	+	—	—	17.9
6	Lactose	+	Iodoacetate	—	31.0
7	Lactose	+	NaF	—	18.4

Each Warburg vessel contained 0.5 ml. extract and 1.0 ml. 0.025 *M* bicarbonate buffer. Quantities and concentrations of other components were: 0.5 ml. 1% sugar, 0.1 ml. 3% Ba salt of ATP, 0.2 ml. 0.04 *M* iodoacetate, 0.2 ml. 0.5 *M* fluoride. The vessel contents were equilibrated with 5% CO<sub>2</sub> in nitrogen. The contents of sidearm 1 were tipped in at 0 minutes, that of sidearm 2 at 120 minutes.



washed cells of actively growing *S. thermophilus* cultures fermented lactose two to four times more rapidly than glucose, while galactose was not fermented. Experiments carried out at 30°C. gave different

TABLE I  
*The Fermentation of Phosphorylated Sugars*

(Cu. mm. CO <sub>2</sub> evolved)					Lactic Acid in γ
Time in min.					
Flask	10	20	60	120	
1	4	4	8	13	6.3
2	38	115	293	362	275
3	11	23	67	105	21.6
4	8	11	32	61	16.3
5	8	11	30	57	
6	16	18	21	25	8.8
7	10	11	19	21	

Each flask contained 0.5 ml. bacterial extract. After addition of substrate, the mixture was made up to a volume of 2.5 ml. with 0.025 *M* sodium bicarbonate. Flask 1—control. Flask 2—0.5 ml. 2% α-glucose-1-phosphate. Flask 3—0.5 ml. 2% glucose-1-phosphate + 0.2 ml. 0.5 *M* NaF. Flask 4—1.0 ml. 2.5% α-galactose-1-phosphate. Flask 5—1.0 ml. 2.5% β-galactose-1-phosphate. Flask 6—0.5 ml. 2.3% sodium β-glycerophosphate. Flask 7—0.5 ml. 1% lactose.

TABLE II  
*The Hydrolysis of Lactose by Extract of S. thermophilus*

Tube	Sugar	Mg. sugar per ml.	
		Before incubation	After incubation
1	Lactose	1.79	1.82
2	Glucose	1.79	1.85
3	—	0.08	0.08
4	Lactose	1.80	>3.8
5	Lactose	1.81	>3.8
6	Glucose	1.78	1.84
7	Glucose	1.77	1.70

Tubes 1 and 2 contained no extract. The others contained 0.5 ml. All tubes contained 0.2 ml. 0.5 *M* NaF. Tubes 4 and 6 contained no ATP. The others contained 0.1 ml. 3% ATP. The volume of each tube was made up with 0.25 *M* bicarbonate buffer to 2.8 ml. The mixtures were made up in small tubes which were incubated at 30°C. for 30 minutes. One ml. aliquots were deproteinized with sodium tungstate and reducing values determined with Shaffer-Hartmann Reagent No. 50. The reducing values for tubes 4 and 5 were those for an equal mixture of glucose and galactose. For Reagent 50 the reducing powers of glucose, galactose and lactose are in the ratio 10 : 8 : 4.

results. After about thirty minutes the rate of fermentation of lactose tended to fall to, or below, that of glucose. An effect of temperature on the permeability of the cell membrane to various sugars has not been ruled out. It cannot be decided at present whether the live cells contain an enzyme system for direct fermentation of lactose which is not present in the extract.

#### ACKNOWLEDGMENT

It is a pleasure to thank Dr. Carl F. Cori for his guidance and advice on this problem.

#### SUMMARY

An extract has been made from dried preparations of *S. thermophilus* which fermented lactose and glucose, but not galactose, to lactic acid when ATP was added. Lactose and glucose were fermented at about the same rate. In the absence of ATP,  $\alpha$ -glucose-1-phosphate was fermented rapidly, but no fermentation occurred with either  $\alpha$ - or  $\beta$ -galactose-1-phosphate.

The presence of lactase in these extracts has been demonstrated, and it seems probable that hydrolysis of lactose precedes phosphorylation and fermentation of the glucose part of the molecule.

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# Succinic Dehydrogenase Activity of Certain Endocrine Tissues of the Rat \*

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## INTRODUCTION

During the last three decades great advances have been made in our knowledge concerning the anatomy, physiology and chemistry of the endocrine glands. However, little is known about the mechanisms by which hormones produce their profound effects. Simultaneously with the advances in endocrinology there have been important contributions to our knowledge of the relation of intracellular anaerobic and aerobic oxidizing enzymes to the release and storage of energy for living processes. The advances in this important field have been discussed in review papers (1, 2, 3).

It is conceivable that hormones might influence intracellular metabolism by affecting the quantity of enzyme synthesized and/or the activity of the enzymes of the kind, for example, which are known to be important in glycolysis and in the Krebs cycle. The reactions involved in this cycle result in the oxidation of pyruvic acid in a step-like manner to carbon dioxide and water with the release of energy.

One of the important enzymes involved in this cycle is succinic dehydrogenase which, in the presence of cytochrome c and cytochrome oxidase, catalyzes the dehydrogenation of succinic acid to fumaric acid (4). As a part of a program developed to determine the relation of certain hormones to the succinic dehydrogenase activity of tissues, we have made a survey of the activity of this enzyme in certain tissues, particularly those of the endocrine glands of albino rats.

## EXPERIMENTAL

*Enzyme.* The "homogenization" method of Potter and Elvehjem (5) for studying tissue oxidations was used in the preparation of the tissues

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which were obtained from rats of the Sprague-Dawley strain. The tissues were dissected out and homogenized immediately, and sufficient water was added to make a 5% homogenate.

The proper amounts of the homogenate were placed in Warburg flasks with the fortified substrate worked out by Potter (6, 7) and allowed to equilibrate in the bath at 38°C. for 10 minutes, after which readings were taken at 10 minute intervals. Approximately 20 minutes elapsed from the time the tissue was removed from the animal until the flasks were placed in the Warburg apparatus at 38°C. The oxygen uptake was obtained by taking readings at 10 minute intervals, usually for 60 minutes, although in most cases the first 4 periods were used in calculating the  $Q_{O_2}$ ; i.e., the oxygen uptake per mg. of dry tissue per hour. The dry weights given in Table II were obtained by drying the tissues at 75°C. for 24 hours.

The fortified substrate solution consisted of 1 ml. of 0.1 *M* phosphate buffer of pH 7.4, 0.3 ml. of 0.004 *M*  $CaCl_2 \cdot 2H_2O$ , 0.3 ml. of 0.004 *M*  $AlCl_3$ , 0.3 ml. of 0.5 *M* sodium succinate of pH 7.4, and 0.2 ml. of  $3 \times 10^{-4}$  *M* cytochrome c. This concentration of cytochrome c was found in our experiments to be sufficient to take care of the dilution effect (7). Sufficient water was added to each of the flasks to make a total volume of 3 ml., and 0.1 ml. of 20% potassium hydroxide was used in the center cups.

*Cytochrome c.* The cytochrome c was made from beef heart muscle according to the method of Keilin and Hartree (8) except that the final product was dialyzed against distilled water instead of 1% sodium chloride solution, as Potter (6) found that sodium chloride inhibits the succinoxidase system. The concentration of cytochrome c was determined by use of the Evelyn photoelectric colorimeter (9).

*Substrate.* A 0.5 *M* solution of Eastman sodium succinate was brought to pH 7.4 before it was used.

## RESULTS AND DISCUSSION

As liver was used in developing the method we have employed for determining succinic dehydrogenase activity (5, 6, 7), we used this tissue in checking our technique. It was found that 0.15 ml. of  $3 \times 10^{-4}$  *M* cytochrome c per flask was sufficient to elicit the maximum activity of the succinic dehydrogenase. However, to assure a safe margin, 0.2 ml. of  $3 \times 10^{-4}$  *M* cytochrome c was used in most of the experiments with liver. This amount was also found to be sufficient for the endocrine tissues. The oxygen uptake was shown to be directly proportional to the amount of tissue or homogenate used for the first 10 minute period, and also for the first four 10 minute periods (Table

TABLE I  
*Oxygen Uptake as Related to Quantity of Tissue*

Kind of tissue	No. of 10 min. periods	Amt. of tissue* ml. 5% homogenate	Oxygen uptake—av. for no. of 10 min. periods indicated
			<i>cu. mm.</i>
Liver	4	0.05	13.4
		0.1	24.3
		0.2	50.0
		0.3	74.1
Pituitary	4-6	0.1	3.5
		0.2	6.8
		0.3	10.5
		0.4	14.6
Thyroid	4	0.2	6.9
		0.3	10.3
		0.4	13.3
		0.5	16.9
Adrenal	4	0.1	9.8
		0.2	19.3
		0.3	28.3
		0.4	38.1
Uterus	6	0.2	6.4
		0.3	9.3
		0.4	12.9
		0.5	15.9
Thymus	5	0.2	6.2
		0.3	9.6
		0.4	12.8

\* The fortified substrate used with the various tissue homogenates is given in the text.

I). The experiments were made with at least two, and usually three, different amounts of homogenate so that each run could be checked as to whether the oxygen uptake was proportional to the amount of tissue reacting.

The liver used in these preliminary experiments was obtained from adult rats which were at least four months old. The range of  $Q_{O_2}$  values

of six runs was from 62.4 for the 4 months old rats to 103.9 for the adult animals. The average  $Q_{O_2}$  value for the six runs was 87.5 (Table II) which agrees with the values obtained by Potter and Schneider (7) under similar conditions. The inhibition of this system by malonate was also tested. It was found (one run) that 0.06 ml. of 0.05 *M* malonate decreased the  $Q_{O_2}$  of 77.8 to 44.1. Thus the values obtained for normal liver tissue, and the fact that the activity was inhibited by malonate, indicate that the experimental technique was satisfactory.

TABLE II  
*Succinic Dehydrogenase Activity of Rat Endocrine Tissues*

Source of animals	Age	Kind of tissue	Dry weight percentage of wet weight	No. of expts	$Q_{O_2}$
					<i>average range</i>
Sprague-Dawley <sup>1</sup>	Adult	Liver	28.7	6	87.5(62.4-103.9)
	26 days <sup>2</sup>	Pituitary	21.3	8	23.4(18.0-30.9)
	26 days <sup>2</sup>	Uterus	17.8	7	18.3(14.1-22.0)
	4 mo.	Adrenal	30.0	3	40.0(37.7-44.4)
	2-6 mo.	Thyroid	16.9	3	25.9(23.5-36.3)
	2-6 mo.	Thymus	21.5	4	18.4(16.5-21.0)
	4-6 mo.	Pancreas	27.9	4	9.5(0.0-17.6)
	1.5-4 mo.	Testes	12.7	2	23.0(20.3-25.8)
	4 mo. or older	Seminal vesicle	23.6	1	4.1
	4 mo. or older	Prostate	25.9	2	7.2(5.6-8.8)

<sup>1</sup> Rats of the same strain from the Zoology and Genetics colonies were also used.

<sup>2</sup> Tissues obtained from assay rats that had received gonadotropin.

The source and age of the rats from which the various endocrine tissues were obtained are given in Table II together with the succinic dehydrogenase content of each of the tissues expressed in terms of  $Q_{O_2}$ . Most of these values are based on the first four 10 minute periods, although in some cases the values are based on the first 2, 3, 4 or 6 periods. The reason for this is that, with certain of the endocrine tissues, the oxygen uptake remained almost constant for as long as 60 minutes. Thus the  $Q_{O_2}$  values were computed for the time (number of 10 minute periods) that the uptake remained constant.

The values given in Table I show that for liver, thymus, pituitary,

uterus, thyroid and adrenal tissues the cu. mm. of oxygen taken up per 10 minutes was directly proportional to the amount of tissue expressed in ml. of homogenate. As only a few determinations were made with testes, prostate, seminal vesicle and pancreas this relationship was not determined.

The  $Q_{O_2}$  values given in Table II show that liver has a much higher succinic dehydrogenase activity than the endocrine tissues. In general, tissues such as pituitary, uterus, thymus and testes have  $Q_{O_2}$  values close to 20 as compared with 87.5 for liver. The activity of pituitary tissue was inhibited by malonate. The  $Q_{O_2}$  was reduced from 23.4 to 16.3 and 12.4 by 0.02 ml. and 0.04 ml. of 0.05 *M* malonate, respectively.

Thyroid tissue from male rats 2 to 5 months old had a  $Q_{O_2}$  of 25.9. On the other hand a  $Q_{O_2}$  of 40 was found for adrenal tissue of 4 months' old male rats which was the highest value obtained for any of the endocrine tissues. It is approximately double the value for pituitary tissue, and is similar to that of the lutein tissue of pregnant rats (10). It is of interest to point out that the high succinic dehydrogenase activity of these two endocrine tissues can be correlated with the steroidal nature of the hormones they secrete, and the similarity of their histology.

The value of 23 for testes is quite similar to that for the pituitary, but it was found that seminal vesicle tissue *excluding the fluid* had very little activity, and the value for prostate was 7.2. The extremely low succinic dehydrogenase activity of seminal vesicle tissue may indicate that this enzyme is not important in the production of its secretion. The  $Q_{O_2}$  values for pancreas were variable as indicated by the wide range, and the average value was low, which confirms the finding of Elliott (11) and Albaum and Potter (12), who showed that the low activity can probably be explained by the action of proteolytic enzymes and ribonuclease. However, in the case of seminal vesicle and prostate gland it seems probable that the low succinic dehydrogenase activity is not due to inhibition or inactivation by proteolytic enzymes, but to the small amounts of the enzyme in these tissues.

As has been indicated in our experiments there are differences in the succinic dehydrogenase activity of certain of the endocrine tissues. There are also differences in activity of the same tissue obtained from animals under different physiological conditions, as has been found for ovarian tissue from pregnant and nonpregnant animals (10). Differences of this kind suggest that the investigation of the effect of specific



hormones on the activity of intracellular enzymes could be of value in elucidating the way in which hormones produce their effects.

In examining the enzyme content of an organ, one must bear in mind the kinds of tissue composing the organ, since there is strong evidence that the oxidizing enzymes are concentrated in the secretory epithelium, while the stroma appears to contain almost none of these enzymes (13), and we have shown (10) that the succinic dehydrogenase content of lutein tissue differed markedly from the remainder of the ovarian tissue. However, the demonstration that various endocrine glands contain appreciable quantities of succinic dehydrogenase suggests that these organs probably obtain their energy by enzymatic mechanisms analogous to those in tissues which have received more detailed study.

### SUMMARY

The succinic dehydrogenase activity of certain endocrine tissues, the uterus, seminal vesicle and prostate of the rat was determined and compared with that of rat liver. It was found that the adrenal gland has an activity of approximately half that of liver and approximately twice that of pituitary, uterus, thyroid, thymus and testes. Pancreas, prostate and seminal vesicle tissues had little activity, the activity of the tissue of the seminal vesicle being least of all.

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# Thermal Destruction of Influenza A Virus Hemagglutinin.

## II. The Effect of pH<sup>1</sup>

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### INTRODUCTION

According to Steinhardt (1) the rate of inactivation of pepsin is proportional to the reciprocal of the fifth power of hydrogen ion activity. Stanley (2) and Best and Samuel (3) reported that tobacco mosaic virus infectivity was stable between pH 2 and pH 9. In following the disintegration of tobacco mosaic virus by means of the ultracentrifuge, Eriksson-Quensel and Svedberg (4) and Wyckoff (5) found a stability range which coincided with that of the infectivity. Lauffer and Price (6) showed that the thermal destruction or disintegration of tobacco mosaic virus protein proceeded more rapidly at pH 7 than at pH 5.8, and, later, Lauffer (7) reported that the rate of disintegration of tobacco mosaic virus protein in 6 *M* urea solutions varies with about the three-halves power of the reciprocal of the hydrogen ion concentration in the pH range from 6 to 8. More recently, Price (8) has described the pH stability range of southern bean mosaic virus as being between pH 6 and pH 8.

The stabilities of the hemagglutinin and infectivity of influenza A, influenza B and swine influenza viruses were studied by Miller (9). His results showed that the pH of maximum stability was between 7 and 8 for the hemagglutinin and infectivity of influenza A and swine influenza viruses but was considerably higher for the activities of influenza B virus. In general, at high pH values, the rate of loss in infectivity was greater than the rate of loss in agglutinin.

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pittsburgh. It was also supported in part by a grant from the Buhl Foundation at the University of Pittsburgh.

It is the purpose of this paper to present results of a study of the destruction of hemagglutinin of the PR-8 strain of influenza A virus at high temperatures in various types of buffers at different pH values.

### MATERIALS AND METHODS

The PR-8 influenza A virus preparations, the source of chicken red blood cells, the method of heating the virus, and the method of measuring agglutinin activity were described by Lauffer and Carnelly (10).

*Buffers:* Six potassium phosphate, four ammonia-ammonium chloride, and three sodium acetate-acetic acid buffers, all of ionic strength of 2.0, covering the pH range from 3.7 to 9.5, were prepared. The pH values of the buffers were verified with a Bailey type hydrogen electrode.

*Equilibration of virus with buffers:* The virus preparations were originally contained in a crude 0.1 M phosphate buffer at about pH 7. To bring the virus into equilibrium with each of the buffers discussed above, 5 cc. of virus suspension was placed in a cellophane casing and dialyzed for at least 24 hours against a solution consisting of 20 cc. of one of the strong buffers discussed above diluted to 200 cc. with water.

### DISCUSSION OF EXPERIMENTAL RESULTS

Inactivation experiments were carried out on the virus preparations at several different temperatures for each of the buffers. The reaction velocity constant was then calculated for each in the manner described by Lauffer and Carnelly (10). This method consists of plotting the reciprocal of the square root of residual activity against time. Twice the slope of the best fitting straight line is taken to be the reaction velocity constant. This procedure provided good characterizations of all the data except those obtained at high pH values, and even there it gave usable approximations.

The experimental results are shown graphically in Fig. 1, where  $\log_{10}$  of the specific reaction rate is plotted against the reciprocal of the absolute temperature, in the manner indicated by the Arrhenius equation, for about half of the buffer-virus systems studied. The remainder of the data were comparable but were excluded in order to avoid confusion in the figure. The data for each case can be fitted reasonably well to a straight line. They are not sufficiently accurate however to permit a decision as to whether or not the slopes of these lines vary with pH. On the assumption that the slope is constant, the energy of activation was calculated, according to the Arrhenius equation, to be about 110,000 calories per mole per degree.

The variation of the reaction rate for the destruction of hemagglu-

tinin with pH at constant temperature was determined by finding the intercept of each graph on Fig. 1 with the vertical line corresponding to a temperature of 55°C. This gave values of  $\log_{10}$  of the specific reaction velocity at 55°C. In Fig. 2, these values are plotted against pH.

It can be seen that there are three vertical scales on Fig. 2. The center scale applies to the data obtained with virus preparation No. 1

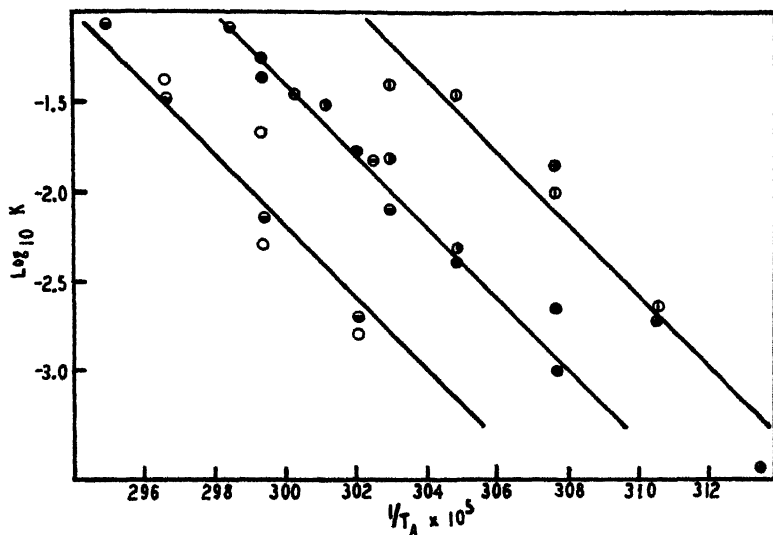


FIG. 1

The Logarithm of the Specific Reaction Velocity for the Destruction of the Hemagglutinin of the PR-8 Strain of Influenza A Virus plotted Against the Reciprocal of the Absolute Temperature.

●—virus preparation No. 1 in phosphate buffer at pH 7.86, ○—same at pH 7.59, ⊖—same at pH 7.15, ⊕—same at pH 6.47, ⊙—virus preparation No. 2 in ammonia buffer at pH 9.55, ●—same in phosphate buffer at pH 6.47, ⊗—same in acetate buffer at pH 5.76.

in phosphate buffers, and the right scale applies to those obtained with virus preparation No. 2 in phosphate buffers and in an acetate buffer. The vertical displacement of the right scale with respect to the center one is a measure of the difference in reactivity of the two virus preparations. This could be due at least in part to differences in concentration (10).

The left scale applies to the data obtained with virus preparation

No. 2 in ammonia buffers. The vertical displacement of the right scale with respect to the left represents a specific difference between the effects upon rates of ammonia and phosphate buffers at the same pH. The data indicate that there is no such difference between phosphate and acetate buffers. In the acetate buffers of pH below 4.5, the rate of destruction of hemagglutinin was so rapid that the activities of the control solutions, held at room temperature, were destroyed.

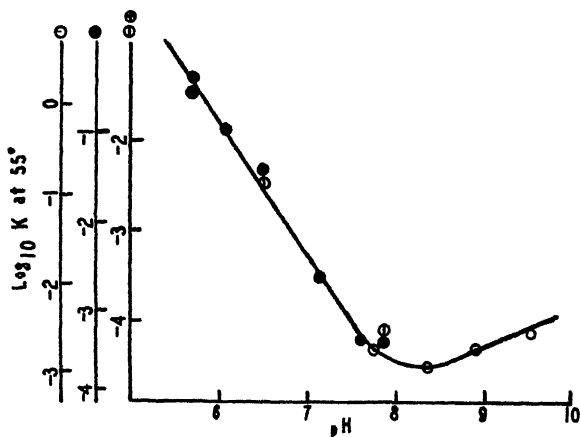


FIG. 2

The Logarithm of the Specific Reaction Velocity at 55°C. for the Destruction of the Hemagglutinin of the PR-8 Strain of Influenza A Virus Plotted as a Function of pH.

○—virus preparation No. 2 in ammonia buffers (left vertical scale), ●—virus preparation No. 1 in phosphate buffers (central vertical scale), ⊙—virus preparation No. 2 in acetate buffer (right vertical scale), ⊕—virus preparation No. 2 in phosphate buffers (right vertical scale).

The data of Fig. 2 show that the rate of thermal destruction of the hemagglutinin of PR-8 influenza A virus at 55°C. has a minimum at pH 8.4. This means that the agglutinin is most stable at 55°C. at pH 8.4. Miller (9) found that the agglutinin of PR-8 influenza A was most stable at 4°C. in the pH range of 7 to 8. At pH 8.6 the agglutinin was still moderately stable, and in one experiment the agglutinin was no more stable at pH 7 than at pH 8.6. We feel, therefore, that Miller's data are not in disagreement with our finding. This leads to the conclusion that the pH value of maximum stability of the agglutinin does

not change appreciably over a temperature range of 50°C. This is as it should be if the data are in agreement with the Arrhenius equation and if the energy of activation does not vary with pH.

It is a little difficult to compare the present data with those obtained for the activities of other viruses. The activity data of Stanley (2) and of Bost and Samuel (3) and the ultracentrifugation data of Eriksson-Quensel and Svedberg (4) and of Wyckoff (5) show that tobacco mosaic virus is fairly stable at low temperatures over the pH range 2-9. The results of Lauffer and Price (6) lead to the conclusion that the pH of minimum rate of disintegration of tobacco mosaic virus protein is at 5.8 or below. Similarly, in the presence of 6 *M* urea the maximum stability of tobacco mosaic virus protein must be below pH 6 (7).

Steinhardt (1) interpreted his finding that the rate of inactivation of pepsin is directly proportional to the reciprocal of the fifth power of hydrogen ion activity to mean that the pepsin molecule must dissociate five hydrogen ions in the process of changing from the normal state to the activated state. If this type of reasoning is carried over to the present case, it is necessary to conclude that the hemagglutinin in PR-8 influenza A virus can proceed to the activated state by two processes, one involving the combination with hydrogen ions and the other involving the dissociation of hydrogen ions. At pH values below 8.4, the former predominates, and at values above 8.4, the latter predominates. It is interesting to observe that the isoelectric point of this strain of influenza virus was found to be 5.3, well below 8.4 (11).

On the basis of theoretical considerations, the effect of pH upon the specific reaction rate of hemagglutinin destruction could be due to an influence upon the energy of activation or upon the entropy of activation. It is possible to calculate by substitutions in the Arrhenius equation that a difference in energy of activation of only 4000 calories per mole per degree could account for the difference between the specific reaction velocity for the destruction of hemagglutinin at pH 6 and pH 8. This magnitude is only about 4% of the average energy of activation for that process. Clearly, the data of Fig. 1 are too inaccurate to enable one to decide whether or not the energies of activation at the extreme pH values differ by as little as 4%. Therefore, it is impossible to decide on the basis of these data whether the effect of pH upon the velocity of this reaction is due to an effect upon the energy of activation or upon the entropy of activation.

## SUMMARY

The rate of inactivation of hemagglutinin of the PR-8 strain of the influenza A virus was studied at various temperatures and in buffers of various pH values. For each pH value, the logarithm of the reaction rate was plotted as a function of the reciprocal of the absolute temperature according to the Arrhenius equation. The energy of activation for this destruction process was found to be about 110,000 calories per mole per degree for all pH values. From the graphs obtained in the manner just described, the variation of specific reaction rate with pH at 55°C. was determined. The rate was found to have a minimum at pH 8.4. It was not possible to decide whether the effect of pH upon the rate of the reaction is due to an effect upon the entropy of activation or upon the energy of activation.

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# Dispensability of Dietary Niacin for Reproduction and Lactation in the Rat

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## INTRODUCTION

A number of investigators have reported that rats will grow in the absence of dietary niacin (1-4). Under such conditions the intestinal flora or the animal's own tissues presumably synthesize sufficient quantities of this nutrient to meet requirements for growth. In the present experiment male and female rats were raised to maturity on synthetic rations deficient in niacin, and the adequacy of such diets for reproduction and lactation determined.

Two basal rations were employed in the present experiment, differing only in their content of synthetic vitamins (Table I). Male and female rats were placed at weaning (21 to 23 days of age) on the above rations. Animals were kept in metal cages with screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib.* feeding. Both males and females were bred at approximately eighty days of age. Females were placed with males of proven fertility, and pregnancy dated from the finding of sperm in the vaginal tract. Males, in turn, were bred to females maintained on a natural food ration adequate for growth, reproduction and lactation. Two strains of rats were employed in the present experiment, hooded rats of the Long-Evans strain and albino animals of the U.S.C. strain.<sup>1</sup>

## GROWTH

Both male and female rats on the above rations made consistent gains in body weight. After sixty days of feeding, males averaged

<sup>1</sup> From the animal colony, Department of Biochemistry and Nutrition, University of Southern California, Los Angeles, California. Kindly provided by Dr. H. J. Deuel, Jr.



239  $\pm$  4 g. and females 178  $\pm$  5 g. on the above rations. No significant difference in growth or gross appearance was observed between female rats of the Long-Evans and U.S.C. strains on any of the diets employed.

### REPRODUCTION

Five of the male rats on diet A and four of those on diet B were bred to females on a natural food ration who subsequently gave birth

TABLE I

*Composition of Experimental Diets and Distribution of Rats*

Dietary Component	Diet A	Diet B
Vitamin Test Casein <sup>1</sup>	22.0	22.0
Salt Mixture <sup>2</sup>	4.5	4.5
Sucrose	73.5	73.5
Vitamin supplements added to diets		
	<i>mg. per cent</i>	<i>mg. per cent</i>
Thiamine hydrochloride	0.2	20.0
Riboflavin	0.4	40.0
Pyridoxine hydrochloride	0.2	2.0
Calcium pantothenate	3.0	20.0
Choline chloride	120.0	120.0
2-Methyl-naphthquinone	0.2	0.5
Distribution of rats		
Female rats (Long-Evans strain)	9	9
Female rats (U.S.C. strain)	12	10
Male rats (U.S.C. strain)	6	6

Each rat on diets A and B also received the following daily supplement: corn oil (Mazola) 500 mg.,  $\alpha$ -tocopherol 0.5 mg., and a vitamin A-D concentrate<sup>3</sup> containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

<sup>1</sup> General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>2</sup> Salt Mixture No. 1, Sure, B., *J. Nutrition* 22, 499 (1941).

<sup>3</sup> Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units vitamin A and 80,000 U.S.P. units vitamin D per gram.

to apparently normal young. Of the females, fifteen were bred on diet A (7 Long-Evans and 8 U.S.C.) and fourteen on diet B (6 Long-Evans and 8 U.S.C.). Twenty-seven of the twenty-nine females bred cast litters; in twenty-four of the litters all young were born alive (average 9.4 per litter). The young were grossly normal with the exception of occasional subepidermal hemorrhagic areas on the tip of the tail and the hind paws (particularly on diet A); average weight of

young at birth was 5.6 g. No significant difference was observed in the reproductive performance of Long-Evans and U.S.C. rats on the above rations, although a greater incidence of hemorrhagic areas was observed in the young of Long-Evans mothers.

### LACTATION

A strain difference was observed in lactation performance between rats of the Long-Evans and U.S.C. strain. Mothers of the Long-Evans strain uniformly failed to nurse their young. Of fifteen litters born to rats of the U.S.C. strain, however, nine were nursed to weaning. Litters were reduced to six each on the day of littering and thirty-nine of fifty-four young were successfully weaned. Young were weaned at twenty-one days of age at an average weight of 31.9 g.

### DISCUSSION

This is the first report to our knowledge concerning the adequacy of niacin-free diets for reproduction in the rat. Results indicate that dietary sources of niacin are dispensable for reproduction in this species, requirements for this factor presumably being met through the synthetic activity of the intestinal flora or the animal's own tissues. A strain difference was observed however as to the adequacy of niacin-free diets for lactation. Whereas nine of fifteen litters born to mothers of the U.S.C. strain on the above rations were nursed to weaning, mothers of the Long-Evans strain uniformly failed to nurse their young. Failure of lactation in the Long-Evans rat does not necessarily indicate a strain difference in niacin requirements for lactation. Under the conditions of the present experiment the intestinal flora or the animal's own tissues synthesized, in addition to niacin, significant quantities of inositol, *p*-aminobenzoic acid, biotin, folic acid and presumably other as yet unidentified nutrients. Strain differences in the synthesis or requirements of the above factors under the conditions of the present experiment may account for the diverse results.

### SUMMARY

Normal reproductive performance was observed in male and female rats raised to maturity on niacin-free diets. A strain difference was observed however in lactation performance; mothers of the U.S.C.

strain successfully nursed their young to weaning, while those of the Long-Evans strain failed to suckle their young.

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## Note on the Orcinol Reagent

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### INTRODUCTION

A confusion exists regarding the orcinol reagent for qualitative identification of sugars which has resulted in a justifiable reluctance on the part of a number of investigators to employ the reagent as a group test for pentoses, uronic acids, pentosans and polyuronides.

This well-known reagent, developed by Bial (1), was incorporated into a coherent scheme of identification by the author (2). After several years of use in the student laboratory, during which hundreds of "unknown sugars" were successfully identified, we ran into difficulty with the orcinol reagent which may account for the discrepancies of others. Part of the difficulty was traced back to the hydrochloric acid employed. In the process of elimination, other obstacles were also discovered. This note is offered to provide directions for consistent results.

### PROCEDURE

Orcinol is dissolved in water in the proportions of 1.0 g. per 100 ml., and kept as a stock solution. Just before use 100 ml. of this solution is mixed with 400 ml. of purest grade concentrated hydrochloric acid and 10 ml. of a 10.0% solution of ferric chloride are added. The solution gives a positive test with all pentoses, pentosans, uronic acids and polyuronides.

Two mg. of the sugar are dissolved in 3 ml. of the reagent and heated in a boiling water bath for 3 minutes. After cooling, 8-10 ml. of *n*-butyl alcohol are added. For visual work it is sufficient to observe the blue to blue-green pigment which is the only acceptable criterion for a positive test. For spectrophotometric comparisons the dilution is continued to 100 ml., or to a suitable depth of color, with *n*-butyl alcohol. Butyl alcohol solutions of the pigment are stable for several hours.

For student laboratories, the reagent can all be mixed at one time and kept for several months without much discoloration. The discoloration does not interfere

with qualitative testing provided sufficient iron has been added and a superior grade of hydrochloric acid has been used.

A Coleman Universal spectrophotometer was employed for the absorption curves.

### DISCUSSION

The usual directions for the preparation of the reagent do not specify sufficient ferric chloride to give positive tests with uronic acids and polyuronides. Pentoses and pure pentosans give good reactions with small amounts of ferric chloride, but apparently uronic acids produce, in acid medium, small amounts of compounds which reduce ferric to ferrous iron. Thus, a greater initial concentration is required. It was never possible to isolate the compound responsible for this

TABLE I

*Additions of Varying Amounts of Iron to 100 ml. of Reagent*

Sugar	Additions in ml. of 10% ferric chloride						
	0.1	0.2	0.3	0.4	0.5	1.0	2.0
Xylose	+	+	+	+	+	+	+
Gum arabic	+	+	+	+	+	+	+
Pectin	-	-	-	-	-	+	+
Calcium glucuronate	-	-	-	-	-	-	+
Barium galacturonate	-	-	-	-	-	+	+
Galacturonic acid	-	-	-	-	-	+	+
Glucurone	-	-	+	+	+	+	+
Calcium 5-ketogluconate	+	+	+	+	+	+	+
Xylan	+	+	+	+	+	+	+
Glucose-xylose mixture	+	+	+	+	+	+	+

+ indicates a positive test; - indicates a negative.

reduction, but it is a well-known fact that uronic acids yield small amounts of enediols. One of these is reductic acid which Reichstein and Oppenauer (3) obtained from glucuronic and galacturonic acids on treatment with dilute sulfuric acid.

In Table I are shown the results from a series of tests in which varying amounts of ferric chloride were added to 100 ml. of reagent. The great difference between the pentoses and the uronic acids is readily seen. Glucurone requires the least iron of the uronics. With calcium glucuronate and barium galacturonate considerable quantities of ferric chloride must be added to obtain positive results.

The importance of the ferric iron is readily observed when the reagent is allowed to stand. After several months it will no longer

give a test with pentoses but, if a small quantity of ferric chloride is added, positive reactions can again be obtained. We have been able to "rejuvenate" in this manner reagents which have stood for a year, even though badly discolored.

Another difficulty in the use of the orcinol reagent has resulted from the various interpretations of what constitutes positive tests. Some manuals and textbooks state that methyloses give a characteristic yellow color and consider this a "positive" reaction. This is an exceptionally poor method to use for the detection of methyloses and should be discarded entirely, as almost any sugar, other than pentoses or uronic acids, can be made to yield a yellow color. Sorensen and Gaugaard (4) have even proposed that the ordinary hexoses be determined quantitatively by means of the orcinol reagent. Then, too, digitoxose supposedly gives a positive reaction (5), because a green color is obtained. This is true, but the green color is in no way comparable with the color obtained by production of furfural, which alone results in the only acceptable positive reaction.

Other investigators have reported success in employing water solutions as the medium for the color identification and comparison. In a recent article, McRary and Slattery (6) and Mejbaum (7) applied orcinol to the quantitative determination of pentoses and pentosans. Their solutions contained only minute amounts of pentose. In our experience, water solutions have proved unsatisfactory due to the limited solubility of the pigment in water and the color of the residual ferric iron. The ferric iron imparts a yellow cast to the water solution which hinders the test in visual observations by pairing with the blue to render the solution green. The actual color of the pigment produced by the pentose sugars is blue. The addition of butyl alcohol to the finished reaction suppresses the iron color so that it does not interfere, and the blue becomes quite evident. This blue color is the only color which should be considered a positive orcinol reaction. Even in the alcohol procedure, the color can be given a greenish cast by caramelizations of other sugars that arise, as an instance, in the hydrolysis of the polysaccharides.

Whenever there is any doubt about the identity of the pigment the spectrophotometer should be employed. In this respect butyl alcohol solutions are superior to water solutions, and we have used them with equal success in qualitative and quantitative work. McRary and Slattery (6) published the absorption spectrum in water solution. The curve in water is a very wide band of absorption which starts at a wave length of 450 m $\mu$  and extends through to a wave length of 750 m $\mu$ . Butyl alcohol solutions give a more precise spectrum and represent absorptions typical of the blue pigment. Fig. 1 shows the absorption

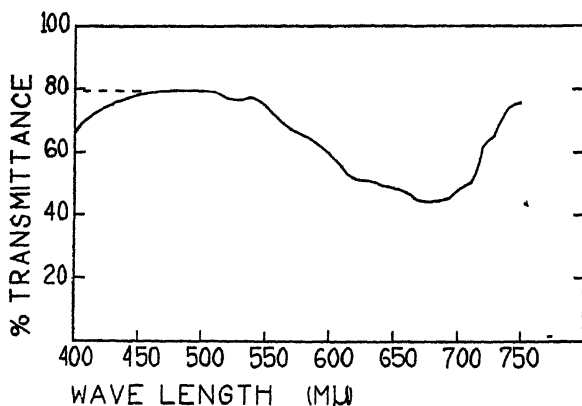


FIG. 1

Absorption Spectrum in *n*-Butyl Alcohol of Xylose Heated with the Orcinol Reagent Containing 0.2 ml. of 10% Ferric Chloride per 100 ml.

in alcohol of a xylose test. The absorption band begins at a wave length of 550  $m\mu$  and extends to 750 with a maximum sufficiently well-developed at 680  $m\mu$  to permit qualitative identification and quantitative determinations. The absorption at 400  $m\mu$  is due to the ferric ion (see Fig. 2, Curve C).

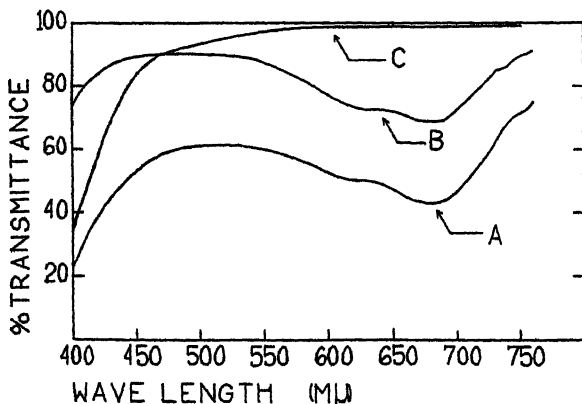


FIG. 2

Absorption Spectrum in *n*-Butyl Alcohol of Orcinol Tests from (Curve A) Pectin, (Curve B) Xylose and (Curve C) Absorption of Ferric Chloride in Butyl Alcohol (reagent strength times 2)

In Fig. 2 the absorption spectrum obtained from pectin is compared to a curve from xylose. The parallelism emphasizes the identity of the pigment obtained from the two tests.

Butyl alcohol solutions of the pigment lend themselves well to quantitative determinations. At the absorption maximum of  $680\text{ m}\mu$  the relation between concentration and optical density is linear. The results in Fig. 3 were obtained by heating 1 ml. of sugar solutions containing 0.5 to 3.0 mg. of arabinose with 3 ml. of orcinol reagent for 5 minutes in a boiling water bath, cooling and diluting to 100 ml. with *n*-butyl alcohol. The optical density of any given sample is deter-

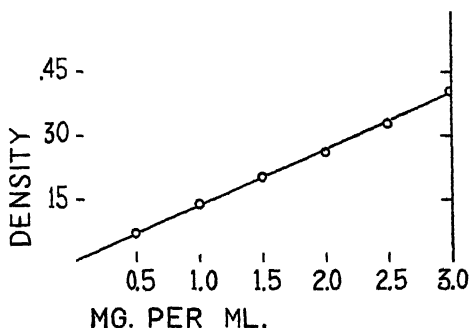


FIG. 3

The Optical Density of Arabinose Solutions Heated with Orcinol Reagent and Diluted with *n*-Butyl Alcohol

mined by the concentration of sugar, the concentration and freshness of the reagent, the duration of heating and the subsequent dilution. By selecting suitable conditions of heating and concentration of reagent an optical density can be obtained which makes possible a quantitative determination of pentoses at almost any concentration. A standard curve must, of course, be run for every sugar, since the rate of furfural formation varies with different carbohydrates.

#### SUMMARY

The efficiency of the orcinol reagent for the identification of pentoses, pentosans, uronic acids and polyuronides is dependent on the purity of reagents, the presence of sufficient ferric iron and the dilution of the reaction mixture with butyl alcohol for observation. The absorp-



tion spectrum of the blue pigment in alcohol is characteristic for carbohydrates producing furfural and can be used for qualitative and quantitative work.

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# The Kiliani Reaction as a Direct Measure of Reducing Groups

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## INTRODUCTION

Since Kiliani (1) first used the addition of hydrogen cyanide to sugars, the reaction has been employed innumerable times to effect syntheses of new sugars and of sugar derivatives. The reaction is quite universal and only in exceptional cases is it incomplete. A complete removal of reducing power results.

Kiliani's failure to exploit the quantitative nature of his reaction most probably originated from the lack of a thoroughly tested method for determination of the excess cyanide. By applying the now well-established Liebig-Dénigès method (2) for estimation of cyanide, the Kiliani reaction is easily made quantitative and becomes a simple and direct measure of the total number of reducing groups in any molecule possessing the aldehyde, ketone, hemiacetal or hemiketal structures. The quantitative application is the subject matter of this publication.

The only other method heretofore available in sugar chemistry which is founded on a stoichiometric equation with reducing groups is the Willstätter-Schudel (3) hypiodite titration and its modifications by Kline and Acree (4) and by Neuberg and associates (5) who employed hypobromous acid. The Willstätter-Schudel reagent reacts only with aldehyde and hemiacetal groups and not with ketone or hemiketal groups. Hydrogen cyanide, however, reacts with all of these, and thus the two methods complement each other for structure determinations.

## PROCEDURE

*Reagents.* 0.1 *N* silver nitrate; 0.2 *N* potassium cyanide; 0.1 *N* acetic acid; 6 *N* ammonium hydroxide.

Two-tenths g. of the sugar to be tested is weighed out and dissolved in 9.00 ml. of 0.1 *N* acetic acid. Ten ml. of the cyanide solution are pipetted into the solution and mixed. The reaction vessel—a glass stoppered 250 ml. Erlenmeyer flask—is

immediately sealed with glycerol and set in a cool place for 24 hours. At the end of this time the stopper is cautiously removed and approximately 10 ml. of 6 *N* ammonium hydroxide are added with a pipette. The stopper is replaced for a few moments, and the flask is shaken to obtain good mixing. Approximately 0.2 g. of potassium iodide is added. The solution is titrated to the first perceptible turbidity with 0.1 *N* silver nitrate. A blank should be run along with the sugar. The difference between the blank and the sample titration times 13 represents the number of milligrams of cyanide as potassium cyanide consumed by the sugar. For 0.2000 g. glucose the theoretical quantity is 72.2.

When working with calcium salts one is likely to run into a precipitate. If this is the case, the calcium should first be removed with oxalic acid and a correspondingly less amount of 0.1 *N* acetic acid added.

With salts of 2-keto acids the reaction time for the Kiliani reaction may be more than 24 hours. Before the titration with silver nitrate this possibility should be checked by running a Fehling's test on the solution.

## RESULTS

In the Liebig method, as improved by Dénigès (2), cyanide is determined by titration in ammoniacal solution with silver nitrate in the presence of silver iodide. The silver cyanide ion is soluble in ammonium hydroxide. Any further addition of silver after the equation is satisfied results in precipitation of silver iodide. The cyanohydrins of sugars produced by the addition of hydrogen cyanide have no effect on the titration, and thus the Liebig-Dénigès method can be used without aspirating the excess cyanide from the solution for subsequent determination.

Table I shows the quantities of cyanide (calculated as potassium cyanide) taken up by a series of sugars. Glucose and galactose were analytically pure and these two sugars conformed exactly to the theoretical requirement of cyanide. The others were run-of-the-shelf samples, but the results are in keeping with the purity of the sugars and, for ordinary work, are quite satisfactory. For determining the number of reducing groups per molecule it is not necessary to use analytically pure compounds.

The error of the method depends upon analytical technique. The usual accuracy is no better than one or two milligrams of potassium cyanide, as small amounts of hydrogen cyanide vapor may be lost easily. During the 24 hour standing hydrogen cyanide establishes a vapor-liquid equilibrium in the flask. The addition of ammonium hydroxide takes up the hydrogen cyanide vapor and prevents any

escape during the titration, but a small loss at the moment the flask is opened becomes unavoidable. Hence a blank is imperative.

The method permits a clear distinction between monosaccharides and disaccharides. Glucose consumes 72.2 milligrams whereas maltose takes up 36.0. Then, too, hexoses can be differentiated from lower carbon monosaccharides; the difference for each carbon increment according to theory is 14.5 milligrams of cyanide. Thus, arabinose requires 85.1 milligrams, whereas glucose requires 72.2.

In combination with the Willstätter-Schudel method a distinction between "aldo" sugars and "keto" sugars can be made. For instance,

TABLE I  
*Consumption of Cyanide as Potassium Cyanide*

Sugar	Observed mgs.	Calculated for anhydrous mgs.
Glucose anhydrous	72.2	72.2
Galactose anhydrous	72.2	72.2
Mannose	73.9	72.2
Fructose	74.2	72.2
Sorbose	70.9	72.2
Calcium-5-keto- <i>D</i> -gluconate	74.1	76.0
Xylose	83.0	86.7
Arabinose	85.1	86.7
Lactose	38.6	38.0
Maltose	36.0	38.0
Cellobiose	36.9	38.0
Sorbitol	0.0	0.0
Sucrose	0.0	0.0

fructose consumes the normal requirement of cyanide for a hexose but does not consume hypiodite as do the isomeric aldohexoses.

The addition of hydrogen cyanide to various sugars proceeds at differing rates. Hence, the 24 hour standing was chosen as a convenient time to allow for completion of reaction. With all compounds thus far tested, with one exception, 24 hours has been sufficient time. The one exception was 2-keto-*D*-gluconic acid. For some unaccountable reason, which is under investigation, 2-keto-*D*-gluconic acid did not react completely on standing for as long a time as 4 to 5 days. This anomaly in behavior was not shown by other 2-keto acids which have been tested and for the present no explanation can be offered.

If there is any doubt concerning the completion of reaction, a

Fehling's or Benedict's test should be made before proceeding with the titration for excess cyanide.

### SUMMARY

The quantity of hydrogen cyanide consumed by a sugar in the Kiliani reaction can be determined by titrating the excess with silver nitrate in the presence of potassium iodide and ammonium hydroxide. Each sugar consumes cyanide in the ratio of one mole for each aldehyde, ketone, hemiacetal or hemiketal group in the molecule.

Various classes of sugars can be differentiated from one another on the basis of cyanide consumption: hexoses from pentoses and tetroses; monosaccharides from disaccharides and trisaccharides.

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# The Production of Riboflavin by *Ashbya Gossypii*

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## INTRODUCTION

Raffy and Fontaine (1) demonstrated the production of large amounts of riboflavin by *Eremothecium ashbyii* Guilliermond when grown on Sabouraud's agar. Guilliermond, Fontaine and Raffy (2) had previously noted a very weak elaboration of this growth factor by the closely related yeast, *Ashbya gossypii* (Ashby and Nowell) Guilliermond. Guilliermond (3) recognized the similarity of the two species. However, since *A. gossypii* existed almost entirely in the mycelial state and never as a budding yeast, and because its ascospores were formed in cells originally plurinucleate instead of mononucleate, he created for it the new genus *Ashbya*. Stelling-Dekker (4) tentatively placed *A. gossypii* with the ascosporogenous yeasts.

In April 1943, we received from Dr. W. J. Robbins, Director of the New York Botanical Gardens, a strain of *A. gossypii* which was incorporated into our culture collection as NRRL Y-1056. As stated by Guilliermond for this species, it existed entirely in the mycelial state. After several days incubation, it produced very pale yellow colonies with a greenish cast. It became more highly colored in serial stock transfers, and late in 1944, there emerged a variant which formed bright orange-yellow colonies within a few days at room temperature. The variant has remained stable for several months of serial transfer on solid media. It is slightly unstable when transferred serially in liquid media, giving rise to cells forming pale or colorless colonies. The fact that this orange-yellow variant produced good yields of riboflavin without the necessity of removing iron from the medium (as shown by Tanner, Vojnovich, and Van Lanen (5) to be required by yeasts of the *Candida* genus) enhances the possible practical importance of *A. gossypii* for the commercial production of riboflavin. This short report is being published in the belief that other investigators may wish to follow up the possibilities of this organism.

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A few preliminary experiments were made to determine the extent of riboflavin production. Two media were used. The first, designated as "yeast extract," had nearly the same composition as the medium used for maintaining stock cultures of yeasts at this laboratory. It was composed of 0.3% powdered yeast extract, 0.5% peptone, and 2% Cerelease (commercial dextrose). The pH was 6.8 to 7.0. The second medium was designated as "Stimuflav," and was the same as the yeast extract medium except that it contained, in addition, 0.3% of Stimuflav.<sup>2</sup> The pH was adjusted to 6.8-7.0 with potassium hydroxide. Both media have been employed as solid substrates by the addition of 2% agar and reduction of the Cerelease content to 1%.

It was found that active aeration led to higher yields of riboflavin than Guilliermond *et al.* obtained with prolonged incubation periods of *E. ashbyii* in still cultures. Aeration was obtained by bubbling air through the medium, or by the continuous agitation of culture flasks on a Ross-Kershaw shaker. Incubation was at 26-28°C. To reduce foaming, cultures through which air was passed received 3 drops of lard oil containing one part per thousand of octadecanol.

One experiment was run to determine the influence of the medium used in preparation of the inoculum on riboflavin yields. Four lines of serial shake cultures were cultivated simultaneously. One line was maintained continuously in yeast extract medium, another in Stimuflav medium, and two were alternately cultivated in the two media. Inocula from yeast extract cultures of the alternating lines always gave higher riboflavin yields than inocula from the line maintained exclusively in yeast extract medium; similarly, inocula from the Stimuflav cultures of the alternating line always gave higher yields than inocula from the line maintained exclusively in Stimuflav medium.

Assays were usually made when the cultures were 4, 6 and 8 days old. Acid extraction, as commonly practiced for liberating riboflavin, was used, because a considerable amount of riboflavin is held inside the cells either as a strongly yellow solution or as crystals (ranging up to a maximum of 16 x 48 micra). The culture was adjusted potentiometrically to pH 1.5 with concentrated sulphuric acid, then autoclaved at 15 pounds for 15 minutes followed by immediate cooling to room temperature and adjustment to pH 5.0 with potassium hydroxide solution. The samples were then filtered and the filtrate assayed for riboflavin with a spectrophotometer.

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<sup>2</sup> Dried distillers solubles manufactured by Hiram Walker and Sons, Inc., Peoria, Illinois. This is not an indorsement of this product over similar products made by other companies.

The effect of aeration rate on riboflavin production by *A. gossypii* grown in yeast extract medium is shown in Table I. The rate is expressed as the number of ml. of air passed through each culture (400 ml.) per minute. The riboflavin yield is given in  $\gamma$  per ml. of medium.

Generally, yields were two to four times as high in cultures through which air was passed as in shake cultures of the same medium grown for the same period of time.

In cultures through which air was bubbled, 1% Cerelose in both yeast extract and Stimuflav media gave greater yields of riboflavin at 4 days than 2% Cerelose, which gave greater yields at 6 days. How-

TABLE I

*Yields of Riboflavin by Ashbya gossypii, NRRL Y-1056, in 2% Cerelose-Yeast Extract Medium Aerated at Various Rates*

Aeration Rate in ml. air/min culture	Analyses						
	4 days			6 days		8 days	
	pH of culture	Dextrose remaining in medium	Riboflavin in $\gamma$ /ml	pH of culture	Riboflavin in $\gamma$ /ml.	pH of culture	Riboflavin in $\gamma$ /ml.
		per cent	$\gamma$		$\gamma$		$\gamma$
ml.							
15	3.6	0.03	77	5.0	156	4.8	152
50	5.0	0.03	53	6.9	153	7.4	283
75	5.0	0.03	70	7.2	217	7.3	381
100	4.5	0.07	30	7.1	73	7.4	163

ever, concentrations of sugar higher than 2% led to progressively lower yields, due partly, at least, to the development of high acidity. Sucrose and maltose, but not lactose, could be substituted for Cerelose.

The addition of 0.3% Stimuflav and 0.1% calcium carbonate to the yeast extract medium increased the yield of riboflavin from approximately 70 to 120–130  $\gamma$  per ml. at 4 days in cultures through which air was bubbled at the rate of 75 ml. of air per minute per 400 ml. of culture.

It should be pointed out that towards the end of the experimental work, a new batch of yeast extract was used. It, unlike the original batch, failed to promote riboflavin production. The new yeast extract appears to be deficient in certain factors, the nature of which will have to be determined by future research.



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# Further Studies on the Metabolism of Nicotinic Acid and Related Compounds in the Horse \*

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## INTRODUCTION

It has been shown (Pearson and Luecke, 1945) that the horse on a minimal daily intake of 0.01 mg. of nicotinic acid per kg. of body weight, or a total of 1.07 mg. daily, excreted in the urine and feces 1.5 to 11.6 mg. of nicotinic acid in excess of the amount ingested. Since the daily excretion of nicotinic acid in the feces and in the urine each exceeded the intake, the synthesis of this vitamin by the horse seems to be well established. It was also reported that, after large oral doses of 5 g. of nicotinic acid daily, 42 to 60% was excreted in the urine and that no extra *N'*-methylnicotinamide, or trigonelline, could be detected in the urine.

The analytical data for nicotinic acid in the paper by Pearson and Luecke were obtained by the microbiological method (Krehl, Strong, and Elvehjem, 1943) employing *L. arabinosus* which responds equally to nicotinic acid, its amide and to nicotinuric acid. Thus, the question of whether or not ingested nicotinic acid is excreted *per se*, as the amide, or as nicotinuric acid remains unanswered. It was, therefore, deemed desirable to extend the earlier studies by making more detailed analyses of the nature of the end products of the metabolism of nicotinic acid, nicotinamide and its methylated derivative, in the horse.

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\*\* Nutrition Foundation Fellow.

## EXPERIMENTAL

Six Shetland ponies about 15 months old and weighing approximately 80 kg. were used in the present studies. The maintenance and dietary regimen of the horses were essentially the same as described previously (Pearson and Luecke, 1945). The horses were fed the basal diet which provided approximately 0.1 mg. of nicotinic acid per kg. of body weight daily, until the urinary excretion of nicotinic acid reached a more or less constant level.

Oral and subcutaneous doses of nicotinic acid, nicotinamide and *N'*-methylnicotinamide were administered for four consecutive days. The urine was collected quantitatively on the third and fourth days. The urine samples were preserved with toluene and HCl.

The total nicotinic acid was determined in the Texas laboratories by the microbiological method (Krehl, *et al.*, 1943) and the preserved samples were shipped by express to Duke University for the chemical analyses. The non-methylated derivatives of nicotinic acid were determined in the unhydrolyzed urine and in the urine after hydrolysis with 1 *N* HCl and 8 *N* HCl as described for human urine by Sarett, Huff and Perlzweig (1942). The *N'*-methylnicotinamide was determined by the recent fluorometric method of Huff and Perlzweig (1945).

The data in Table I indicate that the nicotinic acid in the urine of the horses on the basal ration consists to a considerable extent of derivatives or conjugates yielding nicotinic acid on hydrolysis with HCl. While no attempt was made to isolate and identify the separate fractions, the figures obtained correspond to mixtures of nicotinamide and nicotinuric acid. That this is not the case is shown by the microbiological assay values which are significantly lower than the values obtained after hydrolysis with 8 *N* HCl. Bearing in mind that *N'*-methylnicotinamide is not determined, either in the acid hydrolysates nor in the microbiological assays, it appears that some other hydrolyzable derivative or derivatives of nicotinic acid other than nicotinamide or nicotinuric acid is present in normal horse urine. That these are not likely to be glucuronides was inferred from the fact that the administration of nicotinic acid or of the amide did not affect the excretion of glucuronides in the urine as determined by the method of Hanson, Mills and Williams (1944).

In the previous communication (Pearson and Luecke, 1945) it was reported that it was impossible to detect the presence of *N'*-methylnicotinamide in urines, either normal or after ingestion of nicotinic acid, by the colorimetric methods of Kodicek and Wang (1941) and Sarett (1943). Employing the new fluorometric procedure (Huff and Perlzweig, 1945) by means of which 0.5  $\gamma$  of *N'*-methylnicotinamide (but not trigonelline) can be determined with an accuracy of about

5%, from 3.0 to 4.8 mg. of the methylated derivative was found in the 48-hour urines of the horses on the basal diet. The identity of this compound with *N'*-methylnicotinamide is not certain, although similar

TABLE I

*Urinary Excretion of Nicotinic Acid and Hydrolyzable Derivatives as Determined by the Microbiological Method and Chemical Methods After Various Degrees of Hydrolysis*  
(Values in mg. per 48 hours)

	Animal No	Unhydrolyzed	After 1 N HCl	After 5 N HCl	Microbiological Method	<i>N'</i> -methyl-nicotinamide	Recovery† Per cent
Control—	10	2.3	4.4	7.4	3.9	4.1	
Dietary	55	2.6	5.0	8.2	3.7	3.8	
intake 8 mg.	57	1.9	3.4	5.8	3.3	3.0	
nicotinic	58	2.0	4.1	6.2	2.4	3.5	
acid daily	70	3.5	6.2	10.2	8.8	4.5	
	148	4.4	7.1	11.4	4.4	4.8	
Average		2.8	5.0	8.2	4.4	4.0	
After 4 g.	10	517	567	692	692	2.6	17.1
nicotinic	55	1510	1690	2129	2368	3.3	53.1
acid	57	1800	1920	2170	2352	3.6	54.1
(2 g. daily)	58	1295	1362	1614	1685	5.4	40.2
	70	825	934	1060	1114	4.6	26.3
	148	531	576	741	882	5.0	18.3
Average		1080	1175	1401	1516	4.1	35.0
After 4 g.	10	82	191	271	268	4.9	6.6
nicotinamide	55	42	74	118	96	1.5	2.8
(2 g. daily)	57	71	108	150	128	5.7	3.6
	58	48	103	160	191	3.1	3.9
	70	64	122	182	188	3.9	4.3
	148	46	107	160	186	9.0	3.7
Average		59	118	174	176	4.7	4.1

\* Expressed in terms of nicotinic acid.

† Based on the urinary nicotinic acid derivative as determined by the chemical method.

values were obtained employing an earlier and entirely different fluorometric procedure (Huff and Perlzweig, 1943). We know at present of no urinary component except the pyridine nucleotide

coenzymes † which react in both of the fluorometric methods employed. It is, therefore, possible but not established that the *N'*-methylnicotinamide figures in Table I actually represent the pyridine nucleotides. This point will be discussed later in the paper.

The differences in the urinary products after oral ingestion of nicotinic acid and its amide are very striking. On the third and fourth days of ingesting 2 g. of nicotinic acid daily the horses excreted from 17 to 54% (average 35%), mostly in the unchanged form plus some nicotinuric acid. After ingesting the same amount of nicotinamide they excreted only 2.8 to 6.6% (average 4.1%), mostly as the amide, as shown by the increase in value after hydrolysis with 1 *N* HCl and very little increase after 8 *N* HCl. There was no significant increase over the control in the excretion of *N'*-methylnicotinamide as determined by the fluorometric method. Apparently much more nicotinamide is "destroyed" or changed into unknown compounds than occurs when nicotinic acid is ingested. In spite of the large dosage there was no significant rise in the excretion of the methylated compounds.

To test the possibility of destruction of the nicotinamide by the bacterial flora of the intestinal tract, a single dose of 715 mg. was administered to each of three horses subcutaneously and the 48-hour urines analyzed with the results shown in Table II. It appears that again only 2.5 to 6% of the amide was excreted and about 95% remains unaccounted for. Thus, the possibility of loss and destruction by the intestinal bacteria is largely eliminated. There remains the unexplored possibility of retention of nicotinamide or of a derivative in the tissues.

Since it was previously found that man, rat and rabbit (unpublished data of Huff and Perlzweig) "destroy" orally and parenterally administered *N'*-methylnicotinamide it was thought desirable to test the horse in this respect. In Table II are shown the results of experiments with oral and subcutaneous doses of *N'*-methylnicotinamide. Of the oral dose very little, less than 2%, appeared in the unchanged form while the fate of 98% remains undetermined. It is possible, but not likely, that there was a loss with the feces which were not analyzed. Of the subcutaneously administered dose 45, 50 and 57% was excreted unchanged by the three animals, with approximately one-half re-

† It has recently been established that DPN responds to the fluorometric acetone method for *N'*-methylnicotinamide of Huff and Perlzweig (1945).

maining unaccounted for. There was no evidence in the analytical data that any significant amount of *N'*-methylnicotinamide was demethylated to nicotinic acid derivatives or changed to trigonelline by loss of the amide group. As with the nicotinamide, the possibility of storage in the tissues remains to be explored.

In general, the behavior of the horse in regard to nicotinic acid metabolism is unlike that of man, dog, pig or rat where the chief excretion product is largely *N'*-methylnicotinamide, and appears

TABLE II  
*Urinary Excretion of Nicotinic Acid Derivatives After Administration of N'-Methylnicotinamide and Nicotinamide*

Animal No	Dose	Nicotinic acid Microbiologic		N'-methylnicotinamide		Recovery
		Before	After	Before	After	
		<i>mg per 48 hrs</i>		<i>mg. per 48 hrs</i>		<i>per cent</i>
10	796 mg	3.9	5.1	4.1	19.0	1.9
70	<i>N'</i> -MN*	8.8	5.0	4.5	10.0	0.7
148	Orally	4.4	4.4	4.8	13.0	1.0
10	796 mg	3.9**	4.6	4.1**	453	56
70	<i>N'</i> -MN*	8.8**	5.2	4.5**	358	44
170	Injected	4.4**	5.5	4.8**	398	50
53	715 mg	3.7	20.5	3.8	6.0	2.4
57	Nicotinamide	3.3	28.2	3.0	5.7	3.5
58	Injected	2.4	45.1	3.5	4.4	6.0

\* *N'*-MN—abbreviation for *N'*-methylnicotinamide.

\*\* These values are assumed to be the same in the preceding experiment directly above.

similar to that of the rabbit, the only other herbivorous species studied. However, in common with man and rat, and also rabbit, the horse appears to be capable of "destroying" *N'*-methylnicotinamide. Hence, the possibility cannot be overlooked that the horse (and the rabbit) also methylate nicotinic acid and its amide and destroy the methylated product as rapidly as it is formed in a manner analogous to the predicated formation and transformation of citrulline and ornithine in the Krebs cycle of urea synthesis.

## SUMMARY

The urine of horses maintained on a low (0.1 mg. per kg.) intake of nicotinic acid contains a hydrolyzable derivative of nicotinic acid, apparently not identical with nicotinamide, nicotinuric acid or the glucuronide. It also contains a constituent determinable by methods for *N'*-methylnicotinamide. The possible relation of this product to coenzyme is discussed.

The oral ingestion of 2 g. of nicotinic acid daily leads to the excretion of 18 to 54%, chiefly as such, with small amounts of nicotinuric acid. The oral ingestion and subcutaneous injection of nicotinamide results in the excretion of about 5%, largely as such, while the remaining 95% cannot be accounted for by any of the available methods for nicotinic acid derivatives.

Neither nicotinic acid nor the amide leads to increased excretion of the methylated products, trigonelline or *N'*-methylnicotinamide.

*N'*-methylnicotinamide when administered orally to the horse disappears almost entirely, only about 1% being found in the urine. After subcutaneous injection about one-half is excreted unchanged, the other half could not be detected in the form of any known nicotinic acid derivatives.

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## Note on the Poison Produced by the *Polydesmus* (*Fontaria*) *Vicinus*, Lin.

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### INTRODUCTION

It had been noted that the natives of central Mexico ground a certain species of Myriapoda with plants for poisoning their arrows. This observation prompted an investigation of the active principle of the poison.

The myriapod in question, *viz.*, *Polydesmus* (*Fontaria*) *Vicinus*, Lin., is similar to *R. otomitus*, but is white and much smaller. It shows a small triangular elevation, and its back is more vermiform with the posterior angle of the keels. A shallow groove runs obliquely from the angle of the keel to its posterior border (1) (2) (3). The male of the species, when irritated, produces hydrocyanic acid, which can be detected owing to the strong bitter almond-like odor of the exudate.

### PREPARATION

Ten kg. of the *Polydesmus* were obtained and dried for two hours at 105°C. When cooled, the material was finely pulverized. The powder was washed with petroleum ether until a drop of the ether, after evaporation on filter paper, left no residue. The powder was placed in an Erlenmeyer flask with 100 cc. of alcohol, and the mixture heated to 60°C. This extraction was carried out four times more using 60 cc. of the extractive, five different alcoholic solutions being obtained. The solutions were combined and filtered, first through linen and then through paper. Two to three hundred cc. of a yellow solution were thus obtained. This solution was concentrated and allowed to crystallize in the icebox. The substance was recrystallized from ether, again from alcohol, then treated with Darco in water, and the yield of crystals finally increased by addition of alcohol to the filtrate. Yield, 0.9 g.

The crystals thus obtained are soluble in alcohol, very slightly soluble in ether and cold water, more soluble in hot water. M.P., 196°C.



*Analysis:* Qualitative micro analysis proved the presence of C, H and N, and absence of S and halogen. Quantitative analysis gave the following results (4):

C.....	59.6%
H.....	9.1%
N.....	3.2%
	71.9%

The simplest formula possible is, therefore,  $C_{17}H_{23}O_6N$ .

*Molecular Weight Determination* (5): The determination was carried out by the Rast method. The molecular weight calculated from the empirical formula is 337. The molecular weight found experimentally was 335.2. Therefore, the compound is monomeric, and has the above formula.

#### DETERMINATION OF STRUCTURAL FORMULA

*Hydrolysis of the Compound:* Two g. of the compound were hydrolyzed with dilute sulfuric acid at pH 4 for four hours, and the gas evolved was absorbed in  $AgNO_3$  solution. The precipitate proved to be  $AgCN$ . In addition, an oil layer and a water layer remained in the refluxing flask, which were separated. The oily substance had a boiling point of  $236^\circ C$ .,  $D^{20}_D = 0.9775$ ,  $n^{20}_D = 1.5301$ . This compound contained 81.0% carbon and 7.19% hydrogen. Heated with  $KMnO_4$ , it gave another compound, possibly cumic acid, as its m.p. was  $117^\circ C$ . Since the chemical and physical properties of the oily layer agreed with those of cuminaldehyde described in the literature, it was concluded that the compound in the oily layer was cuminaldehyde. However, for identification, two derivatives were prepared, *viz.*,  $\alpha$ -cuminaldoxime (m.p.  $61^\circ C$ .) and cuminaldehyde semicarbazone (m.p.  $211^\circ C$ .) (6).

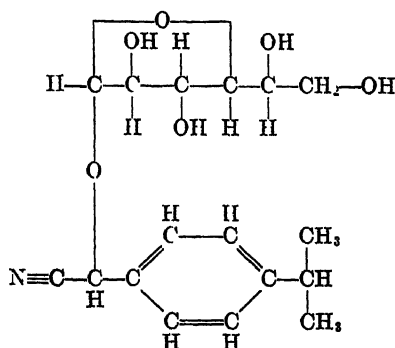
On evaporation of the second layer *in vacuo*, d-glucose was obtained and was identified by its osazone (m.p.  $204^\circ C$ .) and  $\alpha$ -acetate (m.p.  $111^\circ C$ .) (7).

*Determination of the Relation of HCN, Glucose and Cuminaldehyde:* The hydrolysis was repeated with 2 g. and a Volhard titration performed to determine the percentage of HCN present. The cuminaldehyde was removed and weighed and a Fehling determination of the

glucose present was carried out (8). The following percentages of HCN, cuminaldehyde, and glucose were obtained:

HCN .....	7.8%
Cuminaldehyde.....	41.4%
Glucose.....	50.7%
Total.....	99.9%

A calculation would indicate that 1 molecule of HCN, 1 molecule of cuminaldehyde and 1 molecule of glucose are present. Therefore, it is believed that the possible structural formula of the compound would be:



Administration of the substance *per os* to young rabbits (0.2 g. per kg. body weight) causes, at the end of an hour, salivation, constriction of the throat, insensibility, and finally death.

When rabbits are intravenously injected with this substance, they do not die unless emulsin is posteriorly injected.

By enzymatic hydrolysis of the glucoside with emulsin one can prove that HCN, glucose and cuminaldehyde are produced.

#### SUMMARY

1. The Mexican *Polydesmus* (*Fontaria*) *Vicinus*, Lin. when irritated exudes HCN.
2. HCN is combined with glucose and cuminaldehyde.
3. The possible structural formula of the active principle is a glucoside of p-isopropylmandelonitrile.

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# The Application of the Law of Mass Action to Binding by Proteins. Interactions with Calcium

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## INTRODUCTION

A vast literature exists on the combination of proteins with acids, bases, metal ions and neutral molecules (1). In all of this work it is recognized that the protein molecule is capable of combining with more than one of the interacting ions or molecules. In consequence, the law of mass action, as applied to the numerous equilibria which may be involved, becomes quite complex in form. Suitable approaches to the binding of acids and bases by proteins have been worked out by Simms (2), Weber (3), von Muralt (4), and Wyman (5).

On the other hand, the combination of proteins with metal ions or neutral molecules has not been treated adequately from the point of view of the law of mass action.

Recently Greenberg (6) summarized the literature dealing with complexes between alkaline earth cations and proteins, and considered in detail the interactions with calcium. The quantitative treatment of the binding of calcium ions by proteins has been based on the suggestion of McLean and Hastings (7) that the *association* be considered as a single-step process



and that, in formulating an equilibrium constant for the *dissociation* process,

$$\frac{[\text{Ca}^{++}][\text{Pr}^-]}{[\text{CaPr}]} = K_{\text{CaPr}} \quad (2)$$

the protein molecule be treated as a series of independent divalent ions. Thus the number of moles of  $[\text{CaPr}]$  is taken as the difference

between the total calcium and the free calcium

$$[\text{CaPr}] = \text{Ca}_{\text{total}}^{++} - \text{Ca}_{\text{free}}^{++} = \text{Ca}_{\text{bound}}^{++} \quad (3)$$

and the number of moles of  $[\text{Pr}^-]$  is obtained by multiplying the total moles of protein by the maximum number of  $\text{Ca}^{++}$  ions that combine

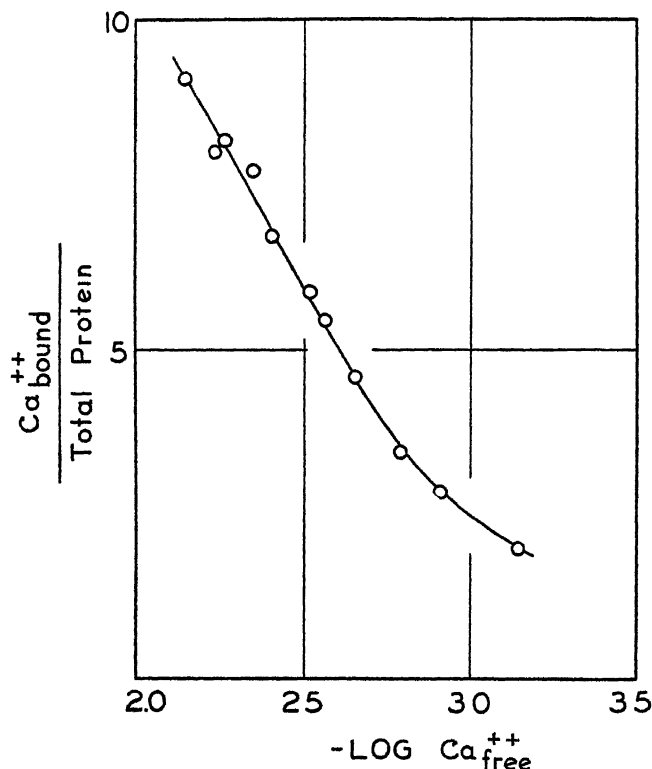


FIG. 1

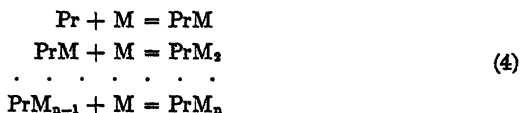
Binding of  $\text{Ca}^{++}$  by Casein as a Function of the  $\text{Ca}^{++}$  Ion Concentration  
(Data of Chanutin, Ludewig and Masket (8))

with one molecule of protein, and then subtracting  $[\text{CaPr}]$ . In estimating the maximum number of  $\text{Ca}^{++}$  ions bound by a protein molecule, McLean and Hastings used the base combining-weight of the protein. A more reliable method, which does not assume that the equivalent weights of protein toward hydroxide ions and calcium ions

are the same, was worked out by Greenberg, Larson and Tufts (8). In either event, however, it becomes immediately evident from an examination of the data of Chanutin, Ludewig and Masket (9) that ten or more calcium ions may combine with each protein molecule and, consequently, that equation (2) is not the proper formulation of the equilibrium constant for such multiple binding. On the other hand, equation (2), and others derived from it are convenient methods of representing the available data, and hence it becomes of interest to consider the conditions under which the law of mass action reduces to these simple forms. To determine these limiting conditions the following analysis has been developed. Inasmuch as the approach is not limited to the binding of  $\text{Ca}^{++}$  ions, and in fact was developed originally to interpret the interactions of drug molecules with proteins, the following equations have been outlined in general form and then applied to the special case of  $\text{Ca}^{++}$ .

#### GENERAL EQUATIONS FOR MULTIPLE BINDING

Consider a molecule or ion, M, which may combine with a protein molecule, Pr (charges are not indicated), in a series of steps:



These reactions may be represented by the classical equilibrium constants, neglecting activity coefficients,

$$\begin{aligned} \frac{[\text{PrM}]}{[\text{Pr}][\text{M}]} &= k_1 \\ \frac{[\text{PrM}_2]}{[\text{PrM}][\text{M}]} &= k_2 \\ &\vdots \\ \frac{[\text{PrM}_n]}{[\text{PrM}_{n-1}][\text{M}]} &= k_n \end{aligned} \quad (5)$$

The following convenient relations can also be derived readily from (5).

$$\begin{aligned} \frac{[\text{PrM}_2]}{[\text{Pr}][\text{M}]^2} &= k_1 k_2 \\ &\vdots \\ \frac{[\text{PrM}_n]}{[\text{Pr}][\text{M}]^n} &= k_1 k_2 \cdots k_n \end{aligned} \quad (6)$$

A useful quantitative expression of the extent of binding is the ratio,  $r$ , of the moles of bound ion or molecule to the total moles of protein. In terms of the quantities introduced in equations (5) and (6),  $r$  becomes

$$r = \frac{[\text{PrM}] + 2[\text{PrM}_2] + \cdots + n[\text{PrM}_n]}{[\text{Pr}] + [\text{PrM}] + [\text{PrM}_2] + \cdots + [\text{PrM}_n]} \quad (7)$$

This equation can be transformed by suitable substitutions from (5) and (6) to give

$$r = \frac{k_1[M] + 2k_1k_2[M]^2 + \cdots + n(k_1k_2 \cdots k_n)[M]^n}{1 + k_1[M] + k_1k_2[M]^2 + \cdots + (k_1k_2 \cdots k_n)[M]^n} \quad (8)$$

A cursory inspection reveals that if  $[M]$  is factored out of the numerator of (8), the remaining factor is merely the derivative of the denominator with respect to  $[M]$ . If we let  $f$  represent the denominator and  $f'$  represent  $df/d[M]$  then equation (8) reduces to

$$r = [M]f'/f \quad (9)$$

#### APPLICATION TO BINDING OF CALCIUM

A typical experimental curve of  $r$  as a function of  $[-\log \text{Ca}_{\text{free}}^{++}]$  is shown in Fig. 1, which has been derived from the data of Chanutin, Ludewig and Masket (9) on casein in solutions of NaOH and NaCl. The range covered by these investigators was from 3 to 18 millimoles of total calcium per kilogram of water. At the highest concentration used, over 9 moles of  $\text{Ca}^{++}$  ion are bound per mole of total protein, as is indicated in Fig. 1. To determine the maximum number of  $\text{Ca}^{++}$  ions which may be bound by one protein molecule, one may plot, as have Chanutin, Ludewig and Masket (9), the ratio of total moles of protein to moles of bound  $\text{Ca}^{++}$  ion against the reciprocal of the free  $\text{Ca}^{++}$  (Fig. 2). The experimental data can then be extrapolated to infinite  $[\text{Ca}_{\text{free}}^{++}]$ , i.e., to  $1/[\text{Ca}_{\text{free}}^{++}] = 0$ , and the reciprocal of the intercept gives the maximum number of  $\text{Ca}^{++}$  ions that may be bound by one molecule of protein. The data of Fig. 2 for casein lead to a figure of 16 for this maximum, with an uncertainty of about  $\pm 1$ .

The McLean and Hastings constant,  $K_{\text{CaPr}}$ , may be reduced now to terms which can be equated to the theoretical relation given in equation (9). From equations (2) and (3) and the related discussion it is obvious that

$$K_{\text{CaPr}} = \frac{[\text{Ca}_{\text{free}}^{++}][m\text{Pr}_{\text{total}} - \text{Ca}_{\text{bound}}^{++}]}{[\text{Ca}_{\text{bound}}^{++}]} \quad (10)$$

where  $m$  is the maximum number of  $\text{Ca}^{++}$  ions bound by one molecule of protein. This equation can be rearranged readily to give the expression

$$\frac{[\text{Ca}_{\text{bound}}^{++}]}{[\text{Pr}_{\text{total}}]} = \frac{m[\text{Ca}_{\text{free}}^{++}]}{K_{\text{CaPr}} + [\text{Ca}_{\text{free}}^{++}]} \quad (11)^*$$

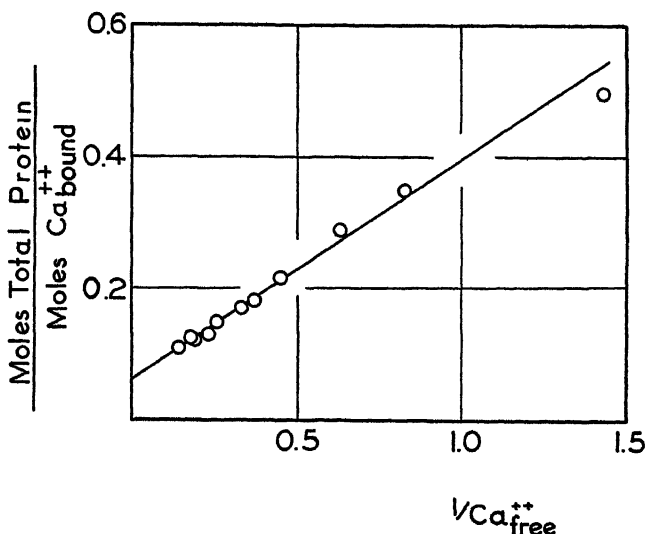


FIG. 2

Extrapolation to Determine the Maximum Possible Number of Bound  $\text{Ca}^{++}$  Ions

which in terms of the generalized notation introduced above leads to the empirical relation for  $r$ ,

$$r = \frac{m[\text{M}]}{K_{\text{CaPr}} + [\text{M}]} \quad (12)^*$$

By equating the relations given in (9) and (12), one can determine the conditions under which the law of mass action reduces to the simple McLean and Hastings expression. Thus, cancelling the common factor, one obtains the equation

$$\frac{f'}{f} = \frac{m}{K_{\text{CaPr}} + [\text{M}]} \quad (13)$$

\* It is of interest to note that this is the form of a Langmuir adsorption isotherm,  $y = ax/(b + x)$ . In other words, expressing data in terms of the McLean and Hastings method amounts to saying that the data can be fitted to a Langmuir isotherm.



This is a very simple differential equation which may be integrated readily to give

$$f = \left( 1 + \frac{[M]}{K_{\text{CaPr}}} \right)^m \quad (14)$$

We are now in a position to evaluate the true mass law constants [equations (5)] in terms of the McLean and Hastings constant,  $K_{\text{CaPr}}$ . Replacing  $f$  by its explicit equation, one obtains

$$1 + k_1[M] + k_1k_2[M]^2 + \cdots + (k_1 \cdots k_n)[M]^n + \cdots + (k_1 \cdots k_m)[M]^m = \left( 1 + \frac{[M]}{K_{\text{CaPr}}} \right)^m \quad (15)$$

Taking casein as an example and assuming that  $m$  is 16 one can differentiate equation (15) successively and obtain a series of sixteen equations of the form:

$$\begin{aligned} k_1 + 2k_1k_2[M] + \cdots + 16(k_1k_2 \cdots k_{15})[M]^{15} &= \frac{16}{K_{\text{CaPr}}} \left( 1 + \frac{[M]}{K_{\text{CaPr}}} \right)^{15}; \\ 2k_1k_2 + (3)(2)k_1k_2k_3[M] + \cdots + (16)(15)(k_1 \cdots k_{15})[M]^{14} &= \frac{(16)(15)}{K_{\text{CaPr}}^2} \left( 1 + \frac{[M]}{K_{\text{CaPr}}} \right)^{14}; \\ \cdots \cdots \cdots &\cdots \cdots \cdots \\ [(15)(14) \cdots (2)(1)](k_1k_2 \cdots k_{15}) + [(16) \cdots (2)](k_1k_2 \cdots k_{15})[M] &= \frac{(16) \cdots (2)}{K_{\text{CaPr}}^{15}} \left( 1 + \frac{[M]}{K_{\text{CaPr}}} \right); \\ 16!(k_1k_2 \cdots k_{15}) &= \frac{16!}{K_{\text{CaPr}}^{16}} \end{aligned} \quad (16)$$

Solving the above equations for the constants defined by equations (6), one obtains

$$\begin{aligned} k_1k_2 \cdots k_{15} &= \frac{1}{K_{\text{CaPr}}^{16}} \\ k_1k_2 \cdots k_{15} &= \frac{16}{K_{\text{CaPr}}^{15}} \\ \cdots \cdots \cdots &\cdots \cdots \cdots \\ k_1k_2 \cdots k_n &= \frac{\frac{16!}{n!}}{(16-n)!K_{\text{CaPr}}^n} \\ \cdots \cdots \cdots &\cdots \cdots \cdots \\ k_1 &= \frac{(16)(15) \cdots (1)}{(15) \cdots (1)} \frac{1}{K_{\text{CaPr}}} = 16 \frac{1}{K_{\text{CaPr}}} \end{aligned} \quad (17)$$

Taking suitable ratios of the equations (17) one may obtain the individual equilibrium constants for the binding of  $\text{Ca}^{++}$  ions by casein:

$$\begin{aligned}
 k_1 &= 16 \frac{1}{K_{\text{CaPr}}} \\
 k_2 &= \frac{15}{2} \frac{1}{K_{\text{CaPr}}} \\
 &\cdot \cdot \cdot \cdot \cdot \cdot \cdot \\
 k_n &= \frac{16 - (n-1)}{n} \frac{1}{K_{\text{CaPr}}} \\
 &\cdot \cdot \cdot \cdot \cdot \cdot \cdot \\
 k_{16} &= \frac{1}{16} \frac{1}{K_{\text{CaPr}}}
 \end{aligned} \tag{18}$$

As an example, numerical values for these constants have been calculated for casein on the basis of a value of 2.23 for  $\text{p}K_{\text{CaPr}}$ , the value derived from the data of McLean and Hastings (7) by Chanutin, Ludwig and Maskot (9). These calculated constants are listed in

TABLE I  
*Equilibrium Constants for Binding of  $\text{Ca}^{++}$  Ions by Casein*

k	Numerical value of k	Numerical value of 1/k	k	Numerical value of k	Numerical value of 1/k
$k_1$	$27 \times 10^2$	$0.037 \times 10^{-2}$	$k_9$	$1.5 \times 10^2$	$0.67 \times 10^{-2}$
$k_2$	13	0.077	$k_{10}$	1.2	0.83
$k_3$	7.9	0.13	$k_{11}$	0.93	1.1
$k_4$	5.5	0.18	$k_{12}$	0.71	1.4
$k_5$	4.1	0.24	$k_{13}$	0.52	1.9
$k_6$	3.1	0.32	$k_{14}$	0.36	2.8
$k_7$	2.4	0.42	$k_{15}$	0.23	4.4
$k_8$	1.9	0.53	$k_{16}$	0.11	9.1

Table I, together with values of their reciprocals, which would be the equilibrium constants for the corresponding *dissociation* processes.

Knowing the individual equilibrium constants one can calculate the concentrations of the various species of bound calcium, *i.e.*,  $[\text{CaPr}]$ ,  $[\text{Ca}_2\text{Pr}]$ , etc., by suitable substitutions in equations (5) or (6). Although the solution of a large number of simultaneous equations is involved, the operations lend themselves to so many simplifications that a complete calculation takes a very short time.

In addition to casein, there are a number of other proteins (7, 9) whose interactions with  $\text{Ca}^{++}$  ions can be correlated conveniently by the McLean-Hastings constant. The true equilibrium constants for these cases may be evaluated readily from the following relation, the generalization of the equations deduced in (18),

$$k_n = \frac{m - (n - 1)}{n} \frac{1}{K_{\text{CaPr}}} \quad (19)$$

where  $m$  represents the maximum number of  $\text{Ca}^{++}$  ions bound by one molecule of protein.

It should be recognized that there are complex situations, such as in blood plasma, where some experimental results (7) may be fitted to a McLean-Hastings constant and hence  $k$ 's evaluated, but these latter constants cannot have the simple significance implied by equations (5), for with more than one protein actively combining with  $\text{Ca}^{++}$  ions, equations (4) are not an adequate description of the equilibria involved. In this connection it is of interest to note that recent work (9) indicates that the binding of  $\text{Ca}^{++}$  by plasma cannot be described by a simple McLean-Hastings constant.

The equations derived here are quite general in nature and can be applied to the interaction of proteins with other ions as well as with molecules. The results obtained in a number of such cases will be described in subsequent publications.

### SUMMARY

1. Equations have been derived from the law of mass action for application to combinations between proteins and ions or molecules.
2. The equations have been applied to the interaction of  $\text{Ca}^{++}$  ions with proteins, for which case they assume a particularly simple form.
3. The data on the combination of  $\text{Ca}^{++}$  with casein have been reviewed, and the true equilibrium constants have been calculated.

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# Electrophoretic Analysis of Swine Plasma and Serum

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## INTRODUCTION

Since the advent of the Tiselius (1) technic for electrophoresis, numerous studies have been made on human and horse plasma and sera. Svensson (2) reported electrophoretic results for swine serum as well as for sera of other animals. More recently Svensson (3) studied the effect of ionic strength on the distribution of albumin and globulin as obtained from electrophoretic diagrams using phosphate buffer pH 7.7 with the addition of NaCl to vary the ionic strength from 0.1-0.47. Cooper (4) made a similar study using barbital buffer at pH 8.6 and 7.8 on artificial protein mixtures and on swine serum. It was the purpose of this investigation to study the behavior of swine plasma and serum in phosphate and barbital (veronal) buffers. Ionic strength was varied to a limited extent, while more emphasis was placed upon comparison of serum with plasma and the efficiency of each buffer for resolution of the components when the plasma or serum was subjected to electrophoresis.

## EXPERIMENTAL

The moving boundary technic of Tiselius (1) as modified by Longworth (5) was used in the electrophoretic determinations. A pooled sample of plasma from 600 swine was used. The plasma was prepared by continuous centrifugation of whole blood to which had been added sodium citrate to a concentration of 0.5% at time of collection on slaughter floor. The plasma was then dried in the frozen state. Electrophoretic determinations were made on a part of the plasma before it was dried. The results were identical with those of the dried plasma. All data reported in this paper are on the dried plasma and fresh serum. The phosphate buffer, pH 7.7, ionic strength 0.2, was prepared according to Green (6). The veronal buffer, pH 8.6, ionic strength 0.1, was prepared according to Longworth (7). The veronal buffer,

pH 8.8, ionic strength 0.2, was made by adding the appropriate amount of NaCl to the buffer of 0.1 ionic strength. The veronal-citrate buffer was made by adding 5.8 g. of sodium citrate to each liter of veronal buffer at pH 8.6, ionic strength 0.1, and then diluting to twice the volume of the original veronal buffer with distilled water. The final pH was 8.2. The veronal-citrate buffer was used to prevent precipitation of fibrinogen during its determinations in the crude fibrinogen preparation.

Approximately 2% protein solutions were made by dissolving the dried plasma in the appropriate buffer. The solutions were then placed in Visking casings and dialyzed against 40 volumes of the buffer. The dialysis was conducted in a 40°F. room for three days with two changes of buffer or until there was no change in the conductivity of the buffer solution. Before making the determination, the protein concentration was checked and adjusted, when necessary, by measuring the index of refraction. Electrolysis of the protein solutions in phosphate buffer was allowed to proceed for 4 hours with a voltage gradient of 4.5 volts per cm., in veronal buffer, ionic strength 0.2, for 4 hours with a voltage gradient of 3.7 volts per cm., and in veronal buffer, ionic strength 0.1, and veronal-citrate buffer, ionic strength 0.1, the electrolysis lasted for 2½ hours and the voltage gradients were 6.2 volts per cm. and 7.4 volts per cm., respectively.

Experiments on fresh swine serum were carried out under conditions identical to the plasma studies. Only two buffers were used—phosphate buffer, pH 7.7, ionic strength 0.2, and veronal buffer, pH 8.6, ionic strength 0.1.

A sample of fibrinogen fractionated from swine plasma was added to whole swine plasma to establish the validity of the electrophoretic method for determining the percentage composition of the various components in the plasma. The fibrinogen was approximately 70% pure as determined electrophoretically in phosphate buffer or 66% as determined in veronal-citrate, ionic strength 0.1 buffer. Traces of albumin and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins were found as impurities. The crude fibrinogen was added so that it formed 10% of the total 2% protein contained in the plasma solution to be electrolyzed. Two buffers were used: namely, phosphate, pH 7.7, ionic strength 0.2, and veronal, pH 8.6, ionic strength 0.1. The plasma and fibrinogen samples were dialyzed separately and then mixed before electrolysis.

Instead of measuring the distance moved by the components from the initial boundary as suggested by Longsworth (8), the distance from the concentration anomaly as suggested by Williams (9) was used. We found mobilities were more consistent when they were based upon the anomaly rather than the initial boundary. Mobilities were calculated on the descending boundaries only for reasons set forth by Longsworth (8).

The concentrations of the protein components in plasma and serum were determined by finding the ratio, in each case, of the component

area to the total area (exclusive of  $\delta$  and  $\epsilon$  boundaries). The areas were measured on projected tracings of both the ascending and descending pictures with a planimeter. The tracings were previously resolved into their components by drawing Gauss curves as Pedersen (10) did in ultracentrifugal analyses and as Longworth (7) later applied to electrophoresis.

## RESULTS

The apparent distribution of the swine whole plasma components separated by electrophoresis in the four buffer solutions used is shown in Table I. Duplicate runs in each solution are reported. The apparent percentage concentrations are given as the average of the ascending and descending boundaries, calculated as described above.

Comparing only the veronal buffer of ionic strength 0.2 and veronal and veronal-citrate of ionic strength 0.1, it is seen that the relative concentrations of  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -globulins and fibrinogen,  $\phi$ , correspond rather closely. In the veronal buffer of ionic strength 0.2 the higher concentration of  $\gamma$ -globulin and the lower concentration of albumin seem significantly different from the corresponding concentrations in the other veronal buffers.

Between the phosphate buffer and the veronal and veronal-citrate buffers there are marked differences. The fibrinogen,  $\phi$ , concentration apparent in the phosphate solution is about one-half the percentage value obtained in the veronal buffers. And conversely, the  $\beta$ -globulin concentration is relatively higher in the phosphate buffer, giving a percentage value of almost twice the  $\beta$ -globulin concentrations appearing in the other buffer solvents. The  $\alpha_1$ -globulin component does not separate out in the phosphate buffer used. The  $\gamma$ -globulin concentrations in phosphate and veronal, ionic strength 0.2, are of the same order, but the phosphate shows an albumin concentration considerably higher than veronal, ionic strength 0.2, and also significantly higher than veronal and veronal-citrate of ionic strength 0.1. The veronal-citrate buffer shows no advantage over the other buffers in preventing the precipitation of fibrinogen in the analysis of crude fibrinogen.

Typical electrophoretic patterns of the swine plasma experiments are shown in Fig. 1. Under the conditions used in these experiments, the veronal buffers of ionic strength 0.2 and 0.1 yield very flat  $\alpha_1$ -globulin boundaries. In all the experiments reported here there is a good separation of the  $\gamma$ -globulin from the  $\delta$  and  $\epsilon$  anomalies, but in



veronal, ionic strength 0.2, the  $\gamma$ -globulin is not adequately separated from the fibrinogen peak. The  $\beta$ -globulin is also poorly resolved from the fibrinogen and  $\alpha_2$ -globulin boundaries in the veronal of higher ionic strength.

ASCENDING BOUNDARY      DESCENDING BOUNDARY



Phosphate Buffer pH 7.7,  $\Gamma/2$  0.2



Veronal Buffer pH 8.8,  $\Gamma/2$  0.2



Veronal Buffer pH 8.6,  $\Gamma/2$  0.1



Veronal-Citrate Buffer pH 8.2,  $\Gamma/2$  0.1

FIG. 1. Whole Plasma

In an ideal electrophoresis the ascending and descending boundary patterns are symmetrical. Longworth has suggested (7) that a ratio

of the maximum refractive index gradient in the descending albumin boundary to that in the rising albumin boundary furnishes an index of this symmetry, the ideal case approaching unity. In the final column of Table I, that ratio is given for each experiment.

For each plasma component obtained electrophoretically in this study, the mobility,  $m$ , is given in Table I.

TABLE I  
*Whole Plasma*

Buffer	pH	I/2	$K_B \times 10^4$	Albumin		$\alpha_1$		$\alpha_2$		$\beta$		$\phi$		$\gamma$		*Symmetry
				$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent	
Phosphate	7.7	0.2	6.72	5.31	38.3			3.94	18.8	3.15	13.2	2.60	11.9	1.75	17.8	.59
Phosphate	7.7	0.2	6.72	5.33	37.9			3.96	18.8	3.18	13.6	2.58	11.9	1.75	17.8	.59
Veronal	8.8	0.2	8.06	5.39	30.5	4.45	4.0	3.81	18.7	2.98	8.5	2.45	20.8	1.68	17.6	.82
Veronal	8.8	0.2	8.06	5.38	30.5	4.42	4.1	3.59	18.9	2.98	8.5	2.40	21.2	1.63	17.0	.87
Veronal	8.6	0.1	3.11	6.28	34.4	5.21	3.9	4.20	18.0	3.45	7.5	2.67	21.5	1.79	14.7	.93
Veronal	8.6	0.1	3.11	6.28	33.5	5.23	4.2	4.20	19.1	3.44	7.7	2.69	20.8	1.82	14.7	.91
Veronal-citrate	8.2	0.1	2.71	6.50	36.5	5.38	3.8	4.47	17.0	3.73	7.9	2.88	21.1	1.86	14.8	.77
Veronal-citrate	8.2	0.1	2.71	6.66	38.7	5.50	4.5	4.57	17.8	3.73	7.7	2.99	19.2	2.03	14.3	.89

\* Symmetry for albumin only.

A sample of fresh swine serum was studied in phosphate, ionic strength 0.2, and veronal, ionic strength 0.1, buffers under the same conditions used in the plasma analysis. The percentage concentration, and mobility,  $m$ , of each resolved component are given in Table II

TABLE II  
*Serum*

Buffer	pH	I/2	$K_B \times 10^4$	Albumin		$\alpha_1$		$\alpha_2$		$\beta_1$		$\beta_2$		$\gamma$	
				$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent	$m$	per cent
Phosphate	7.7	0.2	6.72	5.51	42.4			4.11	18.0	3.22	16.6			1.81	23.0
Phosphate	7.7	0.2	6.72	5.59	42.2			4.18	17.2	3.26	16.4			1.83	24.2
Veronal	8.6	0.1	3.11	6.20	36.9	3.10	3.4	4.10	19.2	3.33	8.3	2.71	12.9	1.97	17.3
Veronal	8.6	0.1	3.11	6.18	37.7	3.10	3.7	4.10	19.4	3.37	7.7	2.78	12.4	1.93	19.1

for duplicate runs in each buffer. A typical boundary pattern in each solvent is shown in Fig. 2.

The apparent concentrations of albumin, and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins in phosphate, ionic strength 0.2, closely approximate the relative

percentages expected if one component, fibrinogen, equal to 12% of the total plasma concentration (as shown in Table I), is completely removed. No such correlation of the serum pattern and the calculated component concentrations can be made with respect to the apparent fibrinogen concentration of whole plasma in the veronal, ionic strength 0.1, buffer. One less peak is anticipated in the serum pattern than is shown in the plasma pattern; due to the removal of fibrinogen, however, the veronal, ionic strength 0.1, buffer yields the same number of peaks, namely six, in both the serum and plasma patterns. It is

ASCENDING BOUNDARY      DESCENDING BOUNDARY

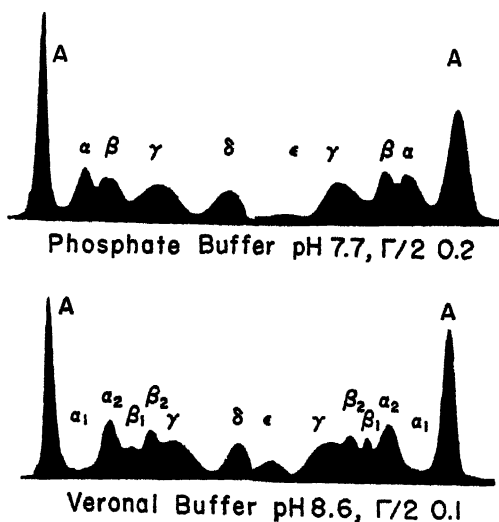


FIG. 2. Serum

interesting to note that the component designated as  $\beta_2$ -globulin of serum in veronal, ionic strength 0.1, Table II, shows a relatively high percentage concentration and has a calculated mobility approximating the mobility of the apparent fibrinogen,  $\phi$ , component of plasma in the same buffer, shown in Table I.

The purpose of the electrophoretic analysis of the plasma and fibrinogen mixture was to test further the behavior of  $\beta_2$ -globulin and fibrinogen in the two buffers. The electrophoretic purity of the fibrinogen in both buffers is shown in Table III and Fig. 3. The results of the plasma-fibrinogen mixture are given in Fig. 4 and Table III.

ASCENDING BOUNDARY

DESCENDING BOUNDARY

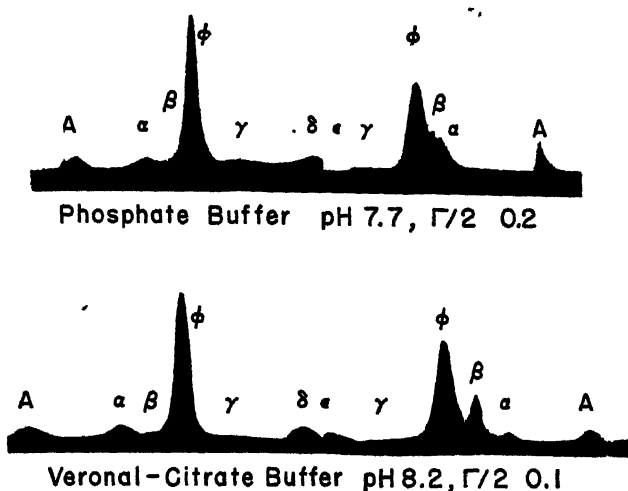


FIG. 3. Fibrinogen

Since the percentage concentration of fibrinogen in whole plasma was found to be 11.9% in phosphate buffer, ionic strength 0.2, Table I, it can be assumed that the fibrinogen contributed by the whole plasma to the plasma-fibrinogen mixture was equal to 10.8% of the total protein concentration. The actual fibrinogen contributed by the 70% pure fibrinogen fraction (Table III) was equal to 7% of the total

TABLE III  
*Fibrinogen and Plasma-Fibrinogen*

Buffer	pH	$\Gamma/2$	$K_B \times 10^3$	Albumin		$\alpha_1$		$\alpha_2$		$\beta$		$\phi$		$\gamma$	
				m	per cent	m	per cent	m	per cent	m	per cent	m	per cent	m	per cent
Phosphate Veronal- citrate				Fibrinogen											
	7.7	0.2	6.02	5.43	10.5			3.40	6.9	3.03	8.7	2.50	69.6	1.49	4.3
	8.2	0.1	2.69	6.45	3.4			4.17	10.2	3.64	11.8	2.84	66.2	1.76	6.4
				Plasma and Fibrinogen											
Phosphate	7.7	0.2	6.72	5.25	35.4			3.87	18.0	3.18	10.5	2.58	18.8	1.62	17.3
Veronal	8.6	0.1	3.11	5.93	30.1	5.05	4.0	4.06	17.2	3.32	7.3	2.55	26.5	1.65	14.9

## ASCENDING BOUNDARY

## DESCENDING BOUNDARY

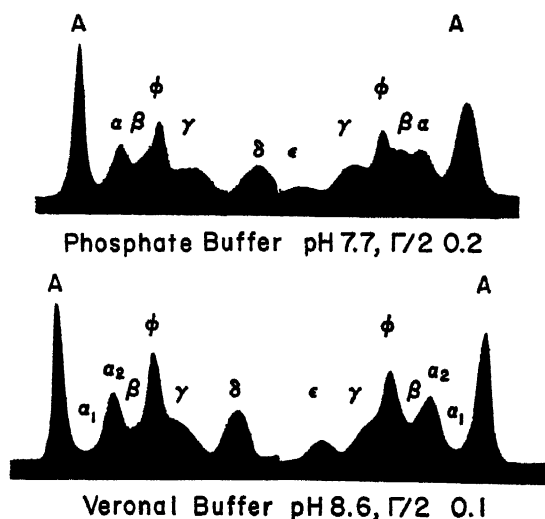


FIG. 4. Plasma and Fibrinogen

protein concentration of the mixture. Therefore, the anticipated percentage concentration of fibrinogen in the plasma-fibrinogen mixture was approximately 18%. The percentage concentration upon analysis was found to be 18.8 (Table III). Similarly, the anticipated percentage concentration of fibrinogen in the plasma-fibrinogen mixture in veronal, ionic strength 0.1, was calculated to be 25.6. The analysis of the mixture in that buffer gave a fibrinogen percentage concentration of 26.5. With respect to the  $\beta$ -globulin in the veronal, ionic strength 0.1, it is of interest that the apparent percentage 7.3, shown in Table III, corresponds with the 8% anticipated concentration calculated in the above manner.

## DISCUSSION

In the electrophoresis of a protein mixture such as plasma, observed in a buffer series of increasing ionic strengths, Svensson (3) predicts a decrease in the relative concentration of the fastest component, albumin, and subsequent increase of the slower ones. Although our observations are limited, the comparison of swine plasma in veronal, ionic strengths 0.1 and 0.2, seems to confirm this.

The considerably higher percentage concentration attributed to albumin in phosphate, ionic strength 0.2, compared to the relative albumin concentrations in the veronal buffers of ionic strengths 0.2 and 0.1, indicates that in phosphate buffer the  $\alpha_1$ -globulin is masked by the albumin peak. Longsworth (7) made a similar observation in phosphate, ionic strength 0.2, in a comparative study of human plasma.

The appearance of a second  $\beta$ -globulin component,  $\beta_2$ , in the electrophoretic pattern of swine serum in veronal, ionic strength 0.1, clarifies the apparent discrepancy in the relative concentrations of  $\beta$ -globulin and fibrinogen in veronal buffers as compared to the phosphate, ionic strength 0.2, results. Since the  $\beta_2$ -globulin in serum is shown to migrate at approximately the same rate observed for fibrinogen under the same experimental conditions, only the faster  $\beta_1$ -globulin component is resolved in the plasma patterns in veronal, ionic strengths 0.2 and 0.1.

The results from the electrophoretic analysis of plasma to which fibrinogen had been added demonstrate that accurate recovery can be expected once the components are resolved and identified.

The mobilities of the various components in each buffer checked remarkably well when plasma, serum and the fibrinogen sample were analyzed.

The authors are indebted to Professor F. C. Koch and Dr. J. B. Lesh for their encouragement and helpful suggestions during this work.

### SUMMARY

Swine plasma and serum have been analyzed electrophoretically using phosphate, veronal and veronal-citrate buffers.

The resolution of the  $\beta$ -globulin, fibrinogen and  $\gamma$ -globulin in phosphate buffer, ionic strength 0.2, was superior to veronal buffers; however, the resolution of the  $\alpha_1$ -globulin and albumin was superior in veronal buffers. It was also noticed that in veronal buffers two  $\alpha$ -globulins and two  $\beta$ -globulins were resolved; however, the second  $\beta$ -globulin migrated with the fibrinogen. In phosphate buffer only one  $\alpha$ - and one  $\beta$ -globulin were resolved with a possibility that  $\alpha_1$ -globulin migrated with the albumin.

A significant difference in the percentage of albumin was noticed when the plasma was analyzed in veronal buffer, ionic strength 0.2, and veronal buffer, ionic strength 0.1, the latter giving a higher concentration.

Electrophoretic analyses of artificial mixtures of fibrinogen and whole plasma demonstrate that an accurate determination of concentrations can be expected under the conditions used.

Mobilities of the components were in good agreement.

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# Failure to Demonstrate Transmethylation of Homocystine by a Strain of *E. Coli* Requiring Methionine for Growth \*

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## INTRODUCTION

Du Vigneaud and his associates (1) have observed that homocystine can replace methionine in the diet when choline or another source of labile methyl groups is available for the transmethylation of homocystine to methionine. Kohn and Harris (3) have succeeded in "training" a strain of *E. coli* so that it requires methionine for growth. If this organism behaved like the mammalian system studied by Du Vigneaud (1), it should be able to methylate homocystine to methionine and thus allow the organism to grow. This report deals with our experiments with the above strain of *E. coli* attempting to induce the transmethylation of homocystine by choline and other methylating agents. The ability of vitamins and amino acids to affect transmethylation in the presence of homocystine and choline was also studied.

## EXPERIMENTAL

Through the courtesy of Dr. Raymond L. Roepeke of the American Cyanamid Company a strain of *E. coli* (No. 15) which did not require methionine for growth and a variant (No. 1-344) requiring methionine were obtained. The methionine-requiring strain was obtained by the procedure described by Kohn and Harris (3). The basal medium employed by us was a modified SG medium as described by Kohn and Harris (2). This medium consisted of 4 g. of NaCl, 2 g., respectively, of  $(\text{NH}_4)_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ , and glucose dissolved in a liter of distilled water. Instead

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\*\* George Lieb Harrison Fellow and Manfred Wahl Fellow.



of using tap water as a source of salts, 100 mg. each per liter of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MgSO}_4$ , and  $\text{CaCl}_2$  were used. After adjusting to a pH of 7.2 the medium was filtered through paper. Since sterilization by autoclaving resulted in slightly caramelized medium containing some precipitate, we resorted to sterilization by filtration through a sintered glass filter which gave us a water clear medium.

In the presence of the SG medium alone, no growth was observed with the methionine-requiring (M) strain. The parent strain, however, grew readily in this medium. Upon the addition of methionine, however, the M strain grew very readily. The minimum concentration of methionine required for growth was about  $10^{-5} M$ . Growth measurements were made by reading the turbidity of the cultures with the Klett-Summerson photoelectric colorimeter.

Since *E. coli* tends to mutate or dissociate, individual vials of the organisms were lyophilized with milk. To obtain a culture, the contents of one vial were transferred into 5 ml. of extract broth. To eliminate any methionine contamination from the milk, one drop of the resulting suspension was inoculated in a fresh tube of extract broth. The organisms were then incubated for not more than 18 to 20 hours, centrifuged, the supernatant discarded and the sediment suspended in 5 ml. of sterile distilled water. One drop of this suspension was used for inoculation in all our experiments. Controls containing SG medium alone, as well as tubes containing various dilutions of methionine, were run along with each experiment to rule out the possibility of mutants. The observation period was from 24 hours to one week.

No growth was observed when either homocystine or choline alone in concentrations from  $10^{-2} M$  to  $10^{-7} M$  was added to SG medium. Nor was growth observed when choline in dilutions from  $10^{-2} M$  to  $10^{-7} M$  was added to a constant amount ( $10^{-3} M$ ) of homocystine. In a few experiments concentrations of  $10^{-2} M$  homocystine and choline were also used without effect. In order to make sure that neither choline nor homocystine, either singly or together, were inhibitory to growth, control experiments with various concentrations of methionine were tried. No inhibition of growth was observed under these conditions. The possibility also presented itself that, even though methionine synthesis occurred, it was not sufficient to raise the concentration to the basal level (approx.  $10^{-5} M$ ) for growth. Accordingly several experiments were made using subminimal quantities of methionine ( $10^{-6} M$ ) in the presence of various dilutions of homocystine and choline. No growth was observed under these conditions. Experiments were also carried out to determine whether or not homocystine and choline contributed to growth in the presence of methionine in concentrations from  $10^{-5} M$  to  $10^{-3} M$ . No augmentation of growth was observed.

Other methylating agents such as betaine, sarcosine, creatinine and

creatine were tried in concentrations from  $10^{-3}$  *M* to  $10^{-7}$  *M* in the presence of  $10^{-3}$  *M* homocystine. No growth was obtained.

As it was thought that vitamins might conceivably have some effect on transmethylation, inositol, pyridoxine, pantothenic acid, riboflavin, thiamin, nicotinamide, biotin, ascorbic acid, and vitamin K, both singly and together, in concentration of 1 to 2  $\gamma$  per ml., and 0.5  $\gamma$  of *L. casei* factor per ml., were added to SG medium in the presence of a constant amount ( $10^{-3}$  *M*) of homocystine and choline. No growth was observed.

Finally, an experiment was set up with a medium containing the following amino acids in *M*/30 phosphate buffer: alanine, valine, leucine, glycine, proline, hydroxyproline, aspartic acid, glutamic acid, phenylalanine, arginine hydrochloride, histidine hydrochloride, lysine hydrochloride, tyrosine, tryptophane and cysteine hydrochloride with 0.5% glucose.\* This medium was similar to the synthetic medium previously employed by us in the study of the mechanism of action of sulfonamides on *S. aureus* (4). No growth occurred in this medium either alone or when both choline and homocystine were added in concentrations of  $10^{-3}$  *M*. However, on addition of  $10^{-3}$  *M* methionine, ample growth occurred. The addition of B complex vitamins in a concentration of 1  $\gamma$  per ml. as previously described, did not stimulate growth in the amino acid medium alone or when choline and homocystine were added.

## DISCUSSION

The results recorded above do not suffice to distinguish with finality between several possible interpretations. It is possible that the *M* strain of *E. coli* cannot under any circumstances methylate homocystine to methionine, or indeed achieve transmethylation at all. However, such a conclusion would not be warranted without studying a much larger series of potential methylating agents under a variety of conditions. Other processes would also have to be taken into account,

\* Individual preparations of the above amino acids, except tyrosine, in concentrations of 0.01 *M* were added to SG medium and tested singly for their ability to cause the growth of the (*M*) strain. Isoleucine, norleucine, norvaline, serine and threonine were also tested in a similar fashion. Tyrosine was tested in a  $2.5 \times 10^{-4}$  *M* concentration. No growth was observed with any of these amino acids under these conditions. This affords evidence for the specificity of methionine in causing the growth of the (*M*) strain.

such as methylation in the course of lipid, carbohydrate, nitrogen and sulfur metabolism. It is also interesting to note that Harris and Kohn (5), using the parent strain, were unable to substitute homocystine and choline for methionine to demonstrate the antagonistic action of methionine against sulfonamides.

It is also possible that additional growth factors may be necessary before the M strain of *E. coli* can methylate homocystine. In the mammal, transmethylation occurs on an exceedingly complex basal diet. The M strain, however, will grow in a very simple basal medium if supplemented with methionine. So far only choline or other methylating agents have been implicated in the transmethylation mechanism of mammals. However, transmethylation, both in the M strain and in mammals, may conceivably require additional and as yet unrecognized factors. Since the M strain of *E. coli* grows rapidly in a simple medium, it might serve as a convenient tool in searching for such possible factors.

#### SUMMARY

It is concluded that, under the conditions of our experiments, a strain of *E. coli* requiring methionine for growth is not able to methylate homocystine in the presence of choline or other methylating agents nor does the addition of amino acids or vitamins make this reaction possible.\*

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\* Thanks are due to Dr. Y. Subbarow of the Lederle Laboratories for a gift of *L. casei* factor, and to Dr. William Elias of John Wyeth and Bro. for a generous supply of pure homocystine and methionine.

## Book Reviews

**Human Biochemistry.** By ISRAEL S. KLEINER, Professor of Biochemistry and Physiology in the New York Medical College, Flower and Fifth Avenue Hospitals. The C. V. Mosby Company, St. Louis, Mo., 1945. 573 pp. Price \$6.00.

In general, two types of text books of biochemistry have been available. In the first, biochemistry is considered as a pure science, organic and physical chemistry are emphasized, and in the consideration of biological relationships, the human organism is no more important than the simplest form of living being. Such a book is often discouraging to medical students, who are at a loss to understand why a pure science should be presented, when their instructors in the later years of the medical course are interested only in man, in clinical applications of biochemistry. The second type of text emphasizes the clinical aspects of biochemistry and rightly so. Such a text is, however, best suited to a student with a preclinical background in biochemistry and with experience in all the preclinical sciences of the first two years of medicine. It is doubtful whether the first term or first year of the medical curriculum is the proper place for such teaching.

"Human Biochemistry" represents a sincere attempt to avoid overemphasis either on pure biochemistry or the clinical point of view. The author has attempted to make available a fundamental chemical background, adequate for the proper understanding of the clinical work of the last two years of the medical curriculum, and at the same time to illustrate these fundamentals with examples chosen from clinical materials, illustrations which serve to make biochemistry alive to the student. This reviewer believes that, even in a text for medical students, pure biochemistry needs no apology, but nevertheless realizes that too often the medical student clamors for the "practical" before he is adequately equipped with the fundamentals.

The author is, on the whole, successful in his attempt to "humanize" biochemistry for the student of medicine. The clinical applications are, for the most part, well chosen and not beyond the understanding of the first year (first term, in many cases) student. If the presentation suffers in its chemical aspects from such a treatment, many may feel that the end sought justifies the means used.

Helpful and unusual in recent texts in biochemistry is the extensive discussion (14 pages) of milk and lactation. The consideration of chemical characteristics of tissues in a single chapter (17 pages) before specialized metabolism in which these tissues are concerned is taken up is also commendable. The weakest chapter is perhaps that relating to physiological oxidations, but the reviewer knows of no elementary text in which this subject is presented so that the student is adequately informed and interested. A satisfactory presentation of this difficult subject must be anticipated.

It is to be regretted that typographic errors occur too frequently in this, the first edition of a useful book. The Henderson-Hasselbalch equation is referred to as the

Henderson-Hasselbach equation on page 26; the great German organic chemist is Fisher on page 96 and Fischer on the following page; thyroxine is spelled without the final E on page 96, although the correct spelling appears later on page 493 and in the index. Such errors, which are too numerous, are not serious but vexatious to the inexperienced student who uses the book.

More serious is a tendency to equivocal statements in many places. Thus on page 89, we are told that gliadin is of poor nutritive value "because of its lack of lysine" and in the same paragraph that zein is "low in lysine." The tabular data on page 107 show the non-inconsiderable amount of 1.3% of lysine in gliadin and no lysine in zein.

Despite these errors which can be remedied in future editions, the author is to be commended for making available a text which is sure to hold the student's interest and to lead him to explore further, perhaps the primary objective in a text.

HOWARD B. LEWIS, Ann Arbor, Michigan

**Bacteriology and Allied Subjects.** By LOUIS GERSHENFELD, Professor of Bacteriology and Hygiene and Director of The Bacteriological Laboratories at The Philadelphia College of Pharmacy and Science in Philadelphia. Mack Publishing Company, Easton, Pennsylvania, 1945. v + 561 pp.

The text differs from the majority of books on bacteriology in that it covers a much wider field than most bacteriological text books. In addition to the usual range of subjects in bacteriology and immunology, there is a very considerable amount of work on animal parasites, insect control, and other pests of economic significance. In addition, more attention has been paid to laboratory techniques in various aspects of bacteriology and immunology than is common in most bacteriological texts. The wide range of subjects covered has precluded as thorough a treatment of many subjects as might be wished. However, it does bring together into a single text a wide variety of information which will be of value to many individuals who are interested in the broad aspects of public health and sanitary control.

The text is generously documented with references which are in the main well chosen. Such references add materially to the value of the book for the general student. The text is, of course, dated by the inclusion of references. This serves to emphasize the fact that any text is out of date before it is published. This is particularly true in certain fields which are advancing rapidly, such as the field of antibiotics.

The book should be valuable as a reference for all students in the wide field which it covers and should find use as a text in those institutions where a single course covers such a wide field.

I. L. BALDWIN, Madison, Wisconsin

**Bioenergetics and Growth.** By SAMUEL BRODY, Associate Professor, University of Missouri. Reinhold Publishing Co., New York, N. Y., 1945, xii + 1023 pp. Price \$8.50.

This encyclopedic work upon the basic biochemistry that underlies growth, maintenance and even aging of the animal body, should have an important place upon the bookshelf of all students of biology. Over two thousand references to the

literature indicate that the author has read widely and then woven an intricate background for his life research in this vast field.

The work will have special value for nutrition students, food economists and scientists in medical and agricultural colleges. For the nutrition student it presents a condensed treatise upon the physiological chemistry of the better known species of animals. For the food economist it lays a scientific background for the optimum use of products of agriculture in feeding man and his domestic animals.

The work will prove a revelation to the scientists in medical schools. Into their narrowly circumscribed field of physiology dealing mainly with the dog, cat, rat and man, the reading of Brody's work will introduce the vast territories of biochemistry of herbivorous species. Brody has drawn extensively upon the conventional literature of the medical school but he has also covered the huge field of agricultural literature dealing with basic knowledge about the cow, the horse, the sheep and the rabbit. These latter are the forgotten species of comparative anatomy and physiology.

The presentation throughout the text is concise and clear. This work is much easier to read than Brody's host of bulletins issued previously. Many of the graphs and illustrations have been reproduced from these bulletins. The graphs will prove difficult to all but the few rare souls who specialize in this field. The index is extensive but could be much improved in future editions if it gave some means of learning facts about given species without reading the whole book. Thus the index does not even contain the words, "calf," "dog," "sheep," "rabbit" or "cow." However these are minor deficiencies in this impressive review.

C. M. McCAY, Bethesda, Md.

**Outline of the Amino Acids and Proteins.** Edited by MELVILLE SAHYUN, Vice-President and Director of Research, Frederick Stearns & Company. Reinhold Publishing Corporation, New York, New York, 1944. 251 pp. Price \$4.00.

The book consists of a brief editor's preface, a foreword by Prof. C. L. A. Schmidt and twelve chapters and an appendix covering the following subjects in the order given: Discovery of the Amino Acids, Sahyun (28 pages); Proteins: Occurrence, Amino Acid Content and Properties, Schmidt (32); Protein Structure, Bull (11); Hydrolysis of Proteins, Sahyun (10); Synthesis and Isolation of Certain Amino Acids, Carter and Hooper (21); Methods of Analysis for Amino Acids and Proteins, Greenberg (37); Relation of Amino Acids and Their Derivatives to Immunity, Heidelberger (6); Relation of Amino Acids to Biologically Important Products and the Role of Certain Amino Acids in Detoxication, Quick (21); Metabolism of Proteins and Amino Acids, Cahill (18); Intermediary Metabolism of Individual Amino Acids, Cahill (20); Nitrogen Equilibrium and the Biological Value of Protein, Cahill and Smith (4); Amino Acids and Proteins in Nutrition, Womack and Kade (16); List of U. S. Patents Issued on Amino Acids and Related Organic Compounds, Laurence (8). An index of 7 pages is also included. The book is well printed in readable type and compactly bound. The text is enlivened by the inclusion in appropriate places of reproductions of amino acid crystals and photographs and biographical sketches of renowned protein chemists.

For the most part the chapters represent adequate and, within themselves, coherent

presentations of the fundamentals and the present state of our knowledge of each subject. Controversial issues, where they have not been avoided, have at least not been belabored. The chapters are thoroughly documented and accordingly represent valuable and readily accessible sources of the classical as well as the currently important references on each subject. For this reason the book will be valuable for advanced students in protein chemistry and biochemistry and should serve admirably as a source book for advanced teaching in both fields. Although, admittedly, the book does not cover all phases of the subject nor attempt to treat any subject exhaustively, the average protein chemist will find in the book much that is new to him in the chapters dealing with fields in which he is not actively engaged. Furthermore, workers in many of the branches of biological science who are acquainted with the fundamentals of amino acid and protein chemistry but who require in their work a knowledge and understanding of the recent advances in these fields, should welcome this book. Readers who come within this category will find that the material presented in this book is not, in many instances, as timely as that provided in the periodic reviews. However, in contrast to such reviews, they will find that recent developments in each of the subjects discussed have not only been appraised and summarized by experts but that sufficient background information has also been presented to permit a more ready comprehension of the significance of the newer work.

While a majority of the chapters are well written, some discharge better than others the obligations set forth in their titles. This is particularly true of the last six chapters. Of the remaining chapters, the discussion of protein structure is particularly good since it concisely and accurately covers a controversial subject in a clear and engaging fashion. The chapter on the synthesis and isolation of certain amino acids is lucidly presented and its worth is augmented considerably by the inclusion of a summary of the best methods for the synthesis of the naturally occurring amino acids. The chapter on methods of analysis is comprehensive and logically presented with special emphasis upon the analytical methods used in medical biochemistry. Industrial protein chemists should find the appendix a valuable check list for patents in the amino acid and protein field.

In spite of its good points, the book falls considerably short of its intended purpose as stated in the foreword in that it does not appear to be "an elementary text that will serve as an introduction" to the chemistry of the amino acids, peptides and proteins. There are some who believe that an elementary text should begin at a point where the reader can grasp the initial concepts presented and then lead him by a progressive series of simple and logical steps into the intricacies of the subject; that a deliberate attempt should be made to make the subject matter interesting as well as informative and inspirational as well as authoritative; that an elementary text should not require the student to delve too deeply for an understanding of the subject into a mass of references, many of which are more abstract, technical and hence less understandable than the material presented in the book. In the present volume the initial chapters to which the beginner might look for the basic information to prepare him for an understanding of the more complicated discussions of protein chemistry to follow, were either never written or at least they were not included in the book.

GEORGE W. IRVING, JR., Beltsville, Maryland

**Physical Methods of Organic Chemistry.** Vol. I. Editor, ARNOLD WEISSBERGER, Eastman Kodak Co. Interscience Publishers, Inc., New York, N. Y., 1945. vii + 736 pp. Price \$8.50.

Descriptions of methods for measurement of physical constants of organic compounds are widely scattered in the literature which includes many books devoted to special aspects of the subject. This volume and its companion, which is soon to appear, are designed to give a modern treatment of the entire field without being *handbucher*. The Editor and authors alike thus had to struggle between Scylla and Charybdis as so well expressed by J. M. Sturtevant in his article on calorimetry: "The subject of calorimetry is very extensive, and its techniques are usually quite involved. It is therefore not possible to present here a complete discussion of the topic. . . . It is hoped that, . . . the discussion will be found to be sufficiently detailed, and the references to the literature sufficiently inclusive, so that a prospective experimenter will not have great difficulty in starting the process of training and equipping himself for calorimetric work."

Subjects treated, the number of pages in the section, and authors are:

Melting and Freezing Temperatures (46) E. L. Skau and H. Wakeham.  
Boiling and Condensation Temperatures (22) W. Swietoslowski  
Density (38) N. Bauer  
Solubility (28) R. D. Vold and M. J. Vold  
Viscosity (14) H. Mark  
Surface and Interfacial Tension (53) W. D. Harkins  
Parachor (9) G. W. Thomson  
Properties of Monolayers and Duplex Films (42) W. D. Harkins  
Osmotic Pressure (24) R. H. Wagner  
Diffusivity (34) A. L. Geddes  
Calorimetry (124) J. M. Sturtevant  
Microscopy (96) E. E. Jelley  
Crystal Form (30) M. A. Peacock  
Crystallochemical Analysis (24) J. D. II. Dounay  
X-Ray Diffraction (36) I. Fankuchen  
Electron Diffraction (32) L. O. Brockway  
Refractometry (84) N. Bauer and K. Fajans.

The general plan of each article includes a short treatment of underlying theory, description of apparatus, and procedures followed. Each author is long experienced in his field and a reader moderately familiar with the subject will find many useful hints and tricks. Duplication is avoided and related articles such as the four, Microscopy, Crystal Form, Crystochemical Analysis and X-Ray Diffraction, are well integrated.

Methods described are generally those of high accuracy requiring large amounts of a compound. Approximate and micro methods so commonly used in organic work are usually not treated. The articles on density and refractometry, which are among the several including such methods, contain useful tables giving usual accuracy attained and amounts of material required.

The book is recommended to physical and organic chemists who might wish to get



an up-to-date treatment of a particular subject within the limits of the title or become familiar with the broad field in order better to interpret the significance of physical constants. An immense amount of effort went into the work which for the greater part bears the mark of considerable originality and in no sense is a mere rehash of other publications.

STERLING B. HENDRICKS, Beltsville, Md.

**Frontiers in Chemistry, Volume IV, Major Instruments of Science and Their Application to Chemistry.** Edited by R. E. BURKE AND OLIVER GRUMMITT. Interscience Publishers, Inc., New York 1945, 151 pages, \$3.25.

"Frontiers in Chemistry" is a serial publication composed of lectures delivered at Western Reserve University by distinguished scientists from industrial and university laboratories on subjects closely related to chemistry. Volume IV, "Major Instruments of Science and Their Application to Chemistry," is a collection of six two-hour lectures dealing with electron diffraction, electron microscopy, X-ray diffraction, emission spectroscopy, absorption spectroscopy and infrared spectroscopy. The essays are apparently intended to introduce chemists to the general principles and the potential usefulness of these physical specialities. A brief subject index is found at the end.

Chapter I, "Electron Diffraction and the Examination of Surfaces," by Dr. Lester H. Germer of the Bell Telephone Laboratories, contains a very brief treatment of the fundamentals of the experimental technic, a consideration of the fields of usefulness of electron diffraction, and a discussion of the structure of very small particles and very thin films. The similarity to and the important differences from X-ray diffraction are brought out clearly.

The second chapter, "The Electron Microscope and its Applications" by Dr. L. Marton, Associate Professor of Electron Optics at Stanford University, begins with an elementary description of the physical principles involved in electron microscopy. An informative section on the construction of the microscope follows. The final section of the essay deals with selected applications of the electron microscope. The preparation of specimens, studies on colloids and on plastics, chemical reactions observable in the microscope, and researches with metals and with biological materials are considered. A relatively extensive bibliography is appended.

Dr. Maurice L. Huggins, research chemist with the Eastman Kodak Company, is the author of Chapter III, "X-ray Diffraction and its Applications." An elementary exposition of the basic principles and of the general experimental procedures introduces the subject adequately. A courageous rationalization of the complex methods of structure projection is presented. Applications other than structure determination are given consideration. The last pages of the chapter are devoted to specific treatments of the structures of typical elements, of simple inorganic compounds, of complex inorganic compounds and of some organic compounds. The essay is bountifully illustrated with diagrams and photographs.

Chapter IV, "Chemical Spectroscopy," is written by Professor Wallace R. Brode of the Ohio State University. This chapter deals with the emission spectra of the elements and their application to qualitative and quantitative analysis. An elementary discussion of the nature of radiant energy serves as an introduction. This is followed by a brief consideration, at the qualitative level, of the atomistics of spec-

trum development. A more detailed discussion of the application to qualitative chemical analysis and a still more detailed treatment of quantitative analysis conclude the chapter. This essay is also well illustrated with diagrams, photographs, and tables of data.

The same author prepared the fifth chapter, "Application of Absorption Spectra to Chemical Problems." No mention is made of the experimental technique, but the methods of describing the data are considered briefly. The rest of the chapter is devoted to a discussion of the absorption of characteristic resonating units or chromophores, to the damping effects of solvents and of groups within the resonating molecule, and to the effect of conjugation. The usefulness of absorption spectroscopy for structure determination and for the qualitative and quantitative analyses of certain compounds is mentioned briefly.

The final chapter, "The Infrared Spectrometer and its Application," is written by Dr. R. Bowling Barnes, Director of the Physics Division of the Stamford Research Laboratories of the American Cyanamid Company. The essay is begun with a very interesting historical treatment of infrared research. This is followed by a brief description of the electromagnetic spectrum and by a more detailed discussion of the oscillations responsible for the infrared spectrum. Experimental apparatus and techniques are described adequately for the non specialist. The remaining pages are devoted largely to the discussion of typical applications in quantitative and qualitative chemical analysis.

Considered individually, no one of the six essays falls short of excellence. The six chapters have one feature in common; they are not intended for the specialist. Some of them will be valuable to the student who wishes to grasp fundamental principles, and others will be particularly useful to the researcher who wishes to assess the applicability of a technique. However, few of the chapters fill both of these needs and the collection as a whole does not completely fill either. In spite of this limitation, the book is a valuable addition to the chemical literature and can be recommended heartily for the partial satisfaction of the requirements of many readers.

MAX A. LAUFFER, Pittsburgh, Pa.

**The Chemistry of Cellulose.** By EMIL HEUSER, The Institute of Paper Chemistry, Appleton, Wisconsin. John Wiley and Sons, Inc., New York, 1944. iv + 660 pp. Price \$7.50.

This book is indeed a valued addition to the vast literature on cellulose chemistry. It has manifold application in the field of cellulose, for it may serve as a useful text and reference for both the student and teacher in carbohydrate, paper and wood chemistry and it likewise will be of value to the research worker. Its usefulness as a reference text is manifest by its comprehensive bibliography which includes more than 2270 references.

The book is divided into fifteen chapters. The first chapter is a brief introduction and discusses the occurrence and nature of cellulose. Chapter II deals with the microscopic and submicroscopic structure of cellulose fibers and the formation of cellulose in plants. Chapters III, IV, V and VI are extensive presentations of the reactions of cellulose with water, aqueous alkalis, organic bases, ammonia, salt solutions and cuprammonium hydroxide. Chapters VII, VIII and IX are concerned with derivatives

namely cellulose esters, xanthates and ethers. Chapters V, VI, VII and VIII deal with the decomposition of cellulose by oxidation, acids, heat and biological processes. The final two chapters, XIV and XV, present studies of the chain structure and molecular weight of cellulose.

The subject matter is largely treated from the chemical point of view and the author has likewise included hypotheses and conclusions based on his many years of research in this field. Many unsolved problems in cellulose chemistry are included which are of interest to the research worker.

On the whole, any criticism of the book may be considered quite minor. Thus on page eight the author seems to prefer the definition of hemicellulose as that portion of a plant material which is soluble in cold sodium hydroxide solution of 17-18% (by weight). In the light of much recent research on hemicellulose, it appears that most investigators prefer to accept the use of hemicellulose as a group term for the non-cellulosic carbohydrates of the cell walls of plants and wood because it entails less confusion. Further, on page nine the author states that besides lignin, wood tissue, depending upon the species, contains numerous other noncellulosic components such as tannins, phlobaphenes and resins. In view of the large amount of information available and the many industries which are based on the extractives obtained from wood such as olcorcin, wood rosin, tall oil and destructive distillation, it would appear that the author's treatment of the noncellulose constituents in wood is inadequate. Also it would seem that a text book on the chemistry of cellulose should include more information on the various methods used in the preparation of cellulose.

A single author who assumes the responsibility of preparing such an adequate text on cellulose chemistry is to be commended. It deals skillfully with the main facets of the chemistry of cellulose.

ARTHUR B. ANDERSON, Portland, Ore.

# Biological Activity of Oxybiotin in the Chick <sup>1</sup>

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## INTRODUCTION

Recently it has been demonstrated that oxybiotin, the oxygen analogue of biotin, can replace biotin in the nutrition of certain microorganisms (1). It was of interest, therefore, to investigate the ability of this compound to replace biotin in the nutrition of higher animals. In view of the relatively high specificity of other dietary essentials in the animal body, oxybiotin would not be expected to be capable of replacing biotin. The chick is a suitable animal for such experiments because of the ease and rapidity with which deficiency symptoms can be developed (2 to 3 weeks, in contrast with 6 to 9 weeks in the rat). The present paper reports experiments demonstrating that oxybiotin can replace biotin in the nutrition of the chick.

## EXPERIMENTAL AND RESULTS

Day-old White Leghorn cockerels were used throughout this study. Groups of 5 chicks each were placed in screen bottom cages in an animal room maintained at about 80°F. and given the experimental ration and water *ad libitum*. The basal diet, a modification of a ration previously used for biotin studies with the chick (2), consisted of dextrin 56%, Labco vitamin-free casein 30%, salts 5% (3), fortified corn oil <sup>2</sup> 2%, Mazola 3%, solubilized Liver Fraction L <sup>3</sup> 2%, and glycine 2%. To each kilo of ration were added thiamine 8 mg., riboflavin 15 mg., pyridoxine 15 mg., calcium pantothenate 50 mg., nicotinic acid 100 mg.,

<sup>1</sup> Contribution No. 578 from the Department of Chemistry, University of Pittsburgh.

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A preliminary report of these studies appeared in *Arch. Biochem.* 7, 393 (1945).

inositol 1 g., and choline chloride 2 g.<sup>4</sup> To insure the development of a severe biotin deficiency, dried raw egg white was substituted for dextrin to make 10% of the diet for one group. In addition, a commercial chick ration<sup>5</sup> mixed to contain 15% of the egg white was used for several experiments. (One group received the unsupplemented commercial ration to serve as normal controls.) Symptoms and weights were recorded weekly. After two weeks, 20% of the chicks receiving diets containing egg white (Table I) showed deficiency symptoms, while those receiving the basal ration alone did not show symptoms until the end of the third week. The development of deficiency symptoms in chicks fed Diets 2 and 7 was comparable, although the growth of Group 7 chicks was somewhat superior.

The growth, survival, and symptomatology observed in prophylactic experiments with biotin<sup>6</sup> and oxybiotin-supplemented diets are summarized in Table I. No deficiency symptoms were developed by any of the chicks receiving either biotin or oxybiotin even at the lower levels. In contrast, all of the surviving control animals showed definite deficiency symptoms.

In a therapeutic experiment, the 12 most deficient animals from Diet Groups 2 and 7 were separated after 25 days into comparable groups of 6 each and injected intramuscularly every other day with either 4  $\gamma$  of *d*-biotin or 8  $\gamma$  of *dl*-oxybiotin. Within one week the mandibular lesions had disappeared completely and some healing of the feet was noted. After three weeks the hard, scaly, cracked skin on the bottoms of the feet had sloughed off, leaving soft normal skin. At this time edema was still evident in some of the segments of the toes of these chicks. Oxybiotin was as effective in curing deficiency symptoms as biotin when injected at these levels. During the therapy period the control animals gained an average of 51 g., the oxybiotin animals 106 g., and the biotin animals 187 g.

Additional deficient chicks after 40 days on Diets 2 and 7 were divided into two comparable groups and these were injected intra-

<sup>1</sup> To 250 g. of Mazola were added 1 g.  $\alpha$ -tocopherol, 125 mg. 2-methyl-1,4-naphthoquinone and 10 cc. Natola. The Natola, kindly furnished by Parke, Davis & Co., Detroit, supplied 55,000 U.S.P. units of Vitamin A and 11,000 U.S.P. units of Vitamin D per g.

<sup>2</sup> Supplied through the courtesy of Dr. David Klein, Wilson Laboratories.

<sup>4</sup> These vitamins were donated by Merck and Company.

<sup>5</sup> Purina Chick Startena purchased from a local feed store.

<sup>6</sup> Kindly supplied by Dr. Karl Folkers, Merck and Co.

muscularly on alternate days with either 2  $\gamma$  of *d*-biotin or 4  $\gamma$  of *dl*-oxybiotin for a three-week period. At this lower level the biotin animals showed growth response and disappearance of deficiency symptoms equivalent to those at the higher injection level, but the oxybiotin animals failed to respond to this therapy. Apparently 4  $\gamma$

TABLE I

*Growth and Deficiency Symptoms of Chicks Fed Oxybiotin or Biotin*

Diet No.	Diet and supplement (per 100 g. ration)	21 Days				40 Days			
		No. of Chicks <sup>1</sup>	Av. Wt. Gain	Symptoms <sup>2</sup>		No. of Chicks <sup>1</sup>	Av. Wt. Gain	Symptoms	
				Feet	Beak			Feet	Beak
1	Basal	19	55	16 <sup>+</sup>	16 <sup>+</sup>	15	110	24 <sup>+</sup>	18 <sup>+</sup>
2	Basal + 10 g. egg white	20	69	39 <sup>+</sup>	33 <sup>+</sup>	8 <sup>3</sup>	112	15 <sup>+</sup>	11 <sup>+</sup>
3	Basal + 10 $\gamma$ <i>d</i> -biotin	9	58	0	0	8	157	0	0
4	Basal + 20 $\gamma$ <i>d</i> -biotin	9	86	0	0	9	218	0	0
5	Basal + 20 $\gamma$ <i>dl</i> -oxybiotin	8	50	0	0	8	129	0	0
6	Basal + 40 $\gamma$ <i>dl</i> -oxybiotin	9	58	0	0	8	120	0	0
7	Commercial diet + 15 g. egg white	10	94	23 <sup>+</sup>	22 <sup>+</sup>	6 <sup>4</sup>	158	11 <sup>+</sup>	11 <sup>+</sup>

<sup>1</sup> Number indicates survivors from original groups. Twenty chicks started on diets 1 and 2; 10 chicks started on all other diets.

<sup>2</sup> Number reported is sum of individual scores; symptoms scored as follows:

0—normal feet or beak.

+—first open lesion on feet, first incrustation around beak.

2<sup>+</sup>—more lesions of increased severity; moderate incrustation around beak.

3<sup>+</sup>—deep hemorrhagic lesions of feet; heavy incrustation around beak.

<sup>3</sup> Eight chicks removed for therapy experiment after 25 days.

<sup>4</sup> Four chicks removed for therapy experiment after 25 days.

of *dl*-oxybiotin every other day are insufficient to bring about the cure of these severe deficiency symptoms.

The 15 deficient chicks surviving after 40 days on Diet 1 were separated into 3 corresponding groups. (Only about half of this group had developed symptoms as severe as those induced by the diets containing egg white.) Five chicks were continued as controls and 5 were transferred to each of the diets containing the lower levels of biotin and oxybiotin (Diets 3 and 5) for a period of 4 weeks. The

biotin group of animals showed almost complete cure of symptoms, while the oxybiotin group was only partially cured. For this interval the control animals gained an average of 74 g., the oxybiotin animals 111 g., and the biotin animals 162 g.

For the quantitative evaluation of the relative activity of biotin and oxybiotin, Diet 7 was fed to a second lot of cockerels. After one week the surviving chicks were divided on a weight and growth basis into 9 comparable groups, one of which consisted of 10 animals retained as negative controls. Four groups of 9 chicks each were injected intramuscularly every other day for 4 weeks with 0.2  $\gamma$ , 0.6  $\gamma$ , 1.2  $\gamma$ , and 2.0  $\gamma$  of *d*-biotin, respectively. In like manner, 2  $\gamma$ , 4  $\gamma$ , 8  $\gamma$ , and 12  $\gamma$  of *dl*-oxybiotin were administered to groups of 9 chicks each. Table II contains a summary of the average growth response to the various levels of biotin and oxybiotin.

## DISCUSSION

The complete absence of deficiency symptoms in those chicks receiving the oxybiotin supplemented rations (Diets 5 and 6) provides a striking demonstration of the activity of this compound in the animal body. Oxybiotin will prevent the development of deficiency symptoms at a dietary level insufficient to produce a significant growth response (see Table I) in accord with general experience with vitamins. Although 20  $\gamma$  of *dl*-oxybiotin per 100 g. of ration were completely effective in preventing the development of deficiency symptoms, this level permitted only a slight improvement during a period of 4 weeks in chicks already severely deficient. For therapy by intramuscular injections, 8  $\gamma$  of *dl*-oxybiotin on alternate days were completely curative, whereas 4  $\gamma$  were without effect. Apparently 8  $\gamma$  are near the minimum curative dose, although the lower dosage was tested on animals after a longer deficiency period and, therefore, the two tests were not entirely comparable. Since 2  $\gamma$  of *d*-biotin were curative under these conditions, the relative activity of *dl*-oxybiotin in curing deficiency symptoms approximates no more than 25% of that of *d*-biotin.

During these therapy experiments it was consistently observed that the mandibular lesions responded more rapidly than the lesions of the feet. In addition to these general symptoms some other abnormalities were observed. Of 41 animals on deficient diets for three or more weeks,

28 exhibited some degree of "curled toe" and 13 developed "slipped tendon." A number of chicks showed improvement of the "curled toe" condition during therapy with either biotin or oxybiotin.

In general, a growth response procedure is more suitable for assay purposes than prevention or cure of deficiency symptoms, because growth can be accurately measured and the assay is not dependent upon subjective evaluation of symptoms. The satisfactory development of deficiency symptoms and the relatively uniform growth response of chicks fed the Startena plus egg white ration in previous experiments suggested the use of this diet for assay purposes. The close agreement

TABLE II  
*Growth Response During Assay of Oxybiotin*

No. of Survivors	Av. Growth Control Period (One Week)	Dosage	Av. Growth Assay Period (Four Weeks)
	<i>g.</i>	<i>γ/day</i>	<i>g.</i>
7	28	0.1 <i>d</i> -biotin	175
8	27	1.0 <i>dl</i> -oxybiotin	188
8	24	0.3 <i>d</i> -biotin	220
7	28	2.0 <i>dl</i> -oxybiotin	226
8	24	0.6 <i>d</i> -biotin	242
8	24	4.0 <i>dl</i> -oxybiotin	254
6	26	1.0 <i>d</i> -biotin	273
8	26	6.0 <i>dl</i> -oxybiotin	267
7	27	controls	120

in growth response of the paired groups used in this experiment (Table II) provides an excellent basis for an accurate measure of the relative biological activity of *dl*-oxybiotin and *d*-biotin. In Figure 1 the average net growth of the various groups has been plotted from the second through the fourth week of the assay period (*i.e.*, the average growth of each group during this interval minus the average growth of the control group). If the quantities of *dl*-oxybiotin required for gains in body weight equal to those produced by 0.05  $\gamma$ , 0.1  $\gamma$ , 0.2  $\gamma$ , 0.3  $\gamma$ , and 0.5  $\gamma$  of *d*-biotin are estimated from these curves, the average relative activity of *dl*-oxybiotin is found to approximate 17% of that of *d*-biotin.



The graded growth response with increasing dosage of oxybiotin is additional proof of the ability of the chick to utilize oxybiotin in its nutrition. Either oxybiotin is transformed into biotin within the organism or oxybiotin itself can perform the functions normally carried out by biotin. Some microorganisms utilize oxybiotin as such (4), but there is no evidence as yet to indicate whether oxybiotin is

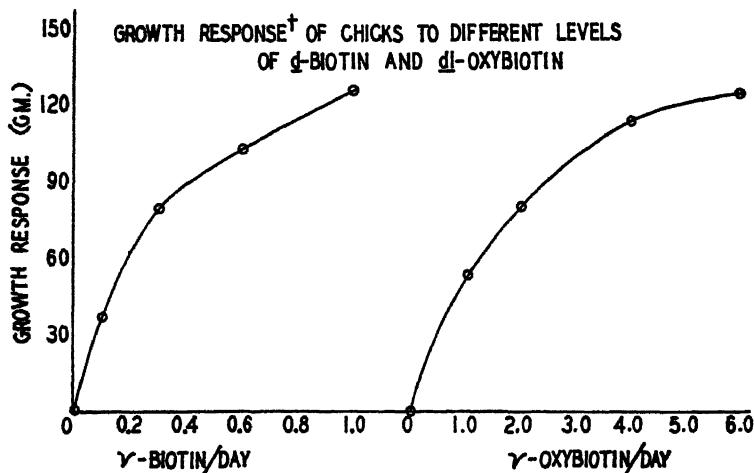


FIG. 1

Difference Between the Average Growth of the Control Group and the Average Growth of Each Experimental Group from the Second Through the Fourth Week of the Assay Period

transformed into biotin by the chick. This question is being investigated at the present time. Regardless of how oxybiotin is utilized, these experiments provide the first example of the replacement of a sulfur-containing compound essential in animal nutrition by its oxygen analogue.

### SUMMARY

Oxybiotin can prevent or cure the symptoms of biotin deficiency in the chick.

Upon the basis of growth response, *dl*-oxybiotin has approximately 17% of the activity of *d*-biotin in the chick.

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# Cobalt Metabolism Studies: Radioactive Cobalt Procedures with Rats and Cattle\*

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## INTRODUCTION

The importance of cobalt as a "trace" element in the diet of livestock is indicated by the fact that diseases apparently attributable to cobalt deficiency have been reported in Florida, Michigan, Massachusetts and New Hampshire as well as in other countries, including New Zealand, Australia, New South Wales, Africa, the United Kingdom and Canada. The present status of knowledge in regard to cobalt metabolism has been reviewed by Russell (1), McCance and Widdowson (2) and Huffman and Duncan (3). It is evident that little is known concerning the role of cobalt in nutrition and its behavior in the animal body.

The cobalt requirement and retention in the animal tissues are so small that the physical and chemical methods of analysis have not been adequate to solve the many fundamental and practical problems involved. It has been necessary, therefore, to turn to the use of radioactive isotopes. This technique yields the basic information that chemical analysis has been able to supply in the case of other nutritionally important substances and, moreover, it is inherently suited for investigating the interrelationships and overlapping factors in mineral metabolism. In 1941 Copp and Greenberg (4) showed the tissue distribution and excretion of labeled cobalt using two rats on a stock laboratory diet; the cobalt was administered to one rat by stomach tube and to the other by intraperitoneal injection. These workers precipitated the cobalt as the sulfide and made the radio-

\* This research was supported by the General Education Board of New York. Published with the permission of the Director of the Florida Agricultural Experiment Station.

activity measurements on this precipitate. In 1943 Greenberg, Copp and Cuthbertson (5) demonstrated the appearance of labeled cobalt in the bile, urine, feces and liver of bile-fistula rats reared on a stock diet.

Although it has been established that cobalt is essential for ruminants there is no clear cut evidence that this element is required by non-ruminant species. For this reason the work has been carried out using "tracer" experiments with cattle as well as with rats. This paper reports procedures developed for the quantitative determination of radioactive cobalt, by means of which large numbers of diversified samples can be handled, and presents observations on the fate of cobalt administered to rats and cattle.

### EXPERIMENTAL

*Radioactive Material:* The radioactive cobalt was supplied by the Massachusetts Institute of Technology—Radioactivity Center and was prepared by bombardment of iron with deuterons. It was obtained as a purified solution of cobalt chloride and consisted of a mixture of three cobalt isotopes ranging from 65 to 270 days half life with the average falling near 80 days.

Two preparations have been received to date for use in this work: *Sample 1*, contained 9 mg. cobalt as cobalt chloride with an initial specific activity of 267  $\gamma$  radium gamma-ray equivalent per mg. *Sample 2*, contained 5.45 mg. cobalt as cobalt chloride with an initial specific activity of 3303  $\gamma$  radium gamma-ray equivalent per mg. In the case of *Sample 1* our measurements were sensitive to 0.004  $\gamma$  cobalt 45 days after its preparation; with *Sample 2* the sensitivity was 0.0006  $\gamma$  cobalt 20 days after its preparation. Since the sensitivity varies with the preparation employed and its age, the corresponding value has been given for each set of data presented.

*Preparation of Tissues:* As quantitative measurements are desired, it is necessary that all the radioactive material in a given sample be brought into a reproducible position with respect to the window of the counter tube. This may be done by ashing the sample, bringing the cobalt into solution and quantitatively electroplating the metal onto a copper disk which is used for the actual measurement (6). These disks are filed for future reference and can be measured as often as desired until such time as the activity is reduced to a low value. Thus, a set of standard disks can be used for calibration purposes over relatively long periods of time.

The following procedure has been found satisfactory for handling most animal tissue samples of 50 to 75 g. or less. The samples are collected in tared porcelain crucibles and, after immediate weighing, are dried at 103°C. so that concentrations can be calculated on the fresh and dry weight basis when desired. To act as a carrier, 1 ml. of a cobalt chloride solution containing 10 mg. inert cobalt is added. After the addition of a few drops of 1:1 nitric acid the samples are ashed at 600°C. for several hours. When filter paper is used, as for the collection of urine, the paper is burned off slowly before the ashing. The ash is dissolved in 2 *N* hydrochloric acid. It was found

necessary to boil the ash in acid for at least two hours, or until the solution was clear, to avoid incomplete recoveries. The acid solution is then transferred to a beaker and evaporated to dryness on a steam bath. Practically all of the acid must be removed at this stage to avoid interference with the electroplating operation which is done in alkaline solution.

The detecting equipment employed at the present time has no scaling circuit and it has been observed that the calibration curve is linear only up to a count of about 400 per minute. It is, therefore, desirable that the amount of activity plated out be adjusted so that the measurement does not exceed this value. To determine whether the total sample can be used, or if an aliquot should be used, a preliminary measurement is made by placing the bottom of the beaker containing the dry residue close to the window of the counter tube and taking the count for 1 minute. The residue is then taken up in a minimum amount of distilled water, and, using plating solution, is made to volume or transferred directly to the plating cell depending on the amount of activity contained.

When the samples contain salts which precipitate in alkaline solution or when very large samples must be used, it is desirable to make a chemical separation prior to the electroplating operation. Satisfactory recoveries were obtained when the following method, according to Mellan (7), was employed. The sample is ashed and brought into solution as already described; this solution is evaporated almost to dryness and about 100 ml. of 5% hydrochloric acid are added. Then 10 ml. of  $\alpha$ -nitroso- $\beta$ -naphthol solution (5 g.  $\alpha$ -nitroso- $\beta$ -naphthol in 75 ml. glacial acetic acid) are added and the mixture heated on a steam bath for an hour and then allowed to stand at room temperature for at least another hour. The solution is filtered, the precipitate washed with water, the filter paper and precipitate placed in a porcelain crucible, and the ashing procedure and subsequent treatment carried out as already described.

Care must be taken to avoid contamination of the samples or glassware with radioactive materials. It is recommended that the glassware be segregated into three groups, one set to be used exclusively with material of high activity such as the original solutions, another set for samples expected to have a medium amount of activity such as excreta, and the other set to be used with samples which experience has shown to contain little or no activity.

*Electroplating of Cobalt:* The body of the electrolytic cell is a piece of pyrex glass tubing with the ends ground square, 25 mm. outside diameter, 1.5 mm. wall thickness, and about 125 mm. in length. The cathode, which forms the bottom of the cell, is a copper disk 25.4 mm. in diameter (disks purchased from the Massachusetts Machine Company, Boston, Mass.). The disks are washed in petroleum ether, dipped in a solution of sulfuric and nitric acids, rinsed thoroughly, dried, and kept protected from dust until used. The disk is fastened to the glass tube with Pyseal sealing wax which makes a leak-proof joint. The cell is mounted in a brass holder.

The plating solution contains 100 g. ammonium sulfate, 180 ml. concentrated ammonium hydroxide, and 5 g. ammonium hypophosphite per liter (6). About 30 ml. of this plating solution containing 10 mg. cobalt and the labeled cobalt to be plated out are used in the electrolytic cell. When the whole sample is used the total cobalt content is essentially 10 mg. since that amount of carrier has been previously added; when aliquots are used, however, a proportional amount of inert cobalt is added to bring the cobalt content to this value.

A Braun Electrolytic Outfit, which accommodates six cells, was found convenient for the plating operation although individual motor stirrers can be used. Platinum anodes serve as stirrers and the current is supplied by storage batteries and regulated by rheostats. Experiment has shown that practically complete recovery is attained by plating at a current density of 27 milliamperes per sq. cm. for  $5\frac{1}{2}$  to 6 hours.

*Measurement of Radioactivity:* The activity is measured with a Geiger-Mueller Counter Apparatus (No. 3 Combination with Type GLB-20 tube purchased from Herbach and Rademan, Inc., Phila., Pa.). The tube consists of a Nonex glass envelope with pyrex end and window: the window is approximately 0.0005 inch thick and the cylinder is 3 cm. in length and 1 cm. inside diameter. It operates at 800 volts. The tube is mounted on bakelite, and a holder provided so that the copper disk may be easily placed in a reproducible position with respect to the tube window. The holder is constructed so that contact is made only with the edge of the disk, upon which no cobalt deposition occurs.

The measuring apparatus is located sufficiently far from the laboratories where the solutions are handled to avoid extraneous counts. The background, due primarily to cosmic radiation, averaged 8 counts per minute for the set-up as described. It is necessary to determine the background count only about twice during the day. Each disk is usually measured for 15 minutes and net counts of 2 per minute or less are considered as not significant. The absolute radioactive cobalt content is obtained by use of standard disks which are prepared by adding known amounts of the radioactive cobalt solution to inert samples and carrying them through the regular procedure as already described. The calibration curve is then constructed by plotting the weight of cobalt against the count per minute. The half life of the cobalt isotopes is such that for practical purposes the calibration standards need be measured only every third day.

## EXPERIMENTAL RESULTS AND DISCUSSION

*Excretion and Tissue Distribution in the Rat:* Inbred Piebald rats, 6 to 9 months old and weighing 150 to 200 g., were used for this work. Some of the animals were reared on a mineral-deficient diet low in cobalt. This ration consisted of 70 g. white corn, 15 g. casein, 3 g. Wesson oil, 0.24 mg. thiamin, 0.1 mg. calcium pantothenate and 0.15 mg. riboflavin; it contained less than 0.06 p.p.m. cobalt as determined by analysis. The animals on the mineral-deficient diet did not grow as rapidly as those on the stock diet; however, this was probably due to a lack of factors other than cobalt. The stock diet contained 0.35 p.p.m. cobalt. In general, pastures containing less than 0.07 p.p.m. cobalt produce deficiency symptoms in cattle, while pastures with more than 0.2 p.p.m. are found to be "healthy" (1).

The animals were fasted for about 18 hours and the radioactive cobalt solution administered quantitatively by stomach tube. The cages were constructed of one-half inch mesh hardware cloth and the

excreta collected on filter paper. It was evident from the data that no detectable contamination of the feces or urine occurred when the collections were made in this manner. The animals were sacrificed by anesthetizing with ether and draining the blood by cardiac puncture, after which the various organs and tissues were removed.

Fig. 1 shows typical excretion curves where the rat was fed 26.4  $\gamma$  labeled cobalt. About 10% of the dose was rapidly eliminated in the urine, and about 80% appeared in the feces within five days, with most of the feces excretion occurring during the first 48 hours. Table I

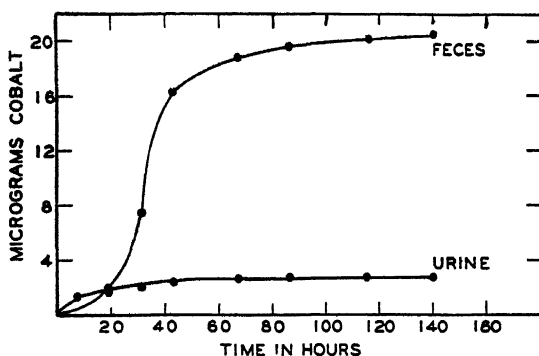


Fig. 1

Excretion of Labeled Cobalt

Inbred Piebald female rat, about 9 months old, weight 130 g.; on low mineral diet, 26.4  $\gamma$  labeled cobalt administered by stomach tube.

presents average excretion values. In this experiment the feces and urine samples were collected until their cobalt content was no longer detectable, which was usually less than 6 days. The cobalt excretion was apparently not affected by the difference in diet. At the 2.2 and 26.4  $\gamma$  levels about 90% of the dose was eliminated, while 70% of the extremely small dose of 0.02  $\gamma$  was excreted. This confirms the contention that the requirement of the rat for cobalt, if there is such, must be very small indeed.

With many of the animals reported in Table I, as well as others, analyses for radioactive cobalt were made on the following tissues: thyroid, adrenals, reproductive tract, brain, eye, lymph glands, salivary glands, heart, blood, lung, trachea, kidney, bladder and contents, tongue, stomach and contents, small intestine and contents, large



intestine and contents, pancreas, spleen, liver, muscle, bone marrow and bones. With the exception of those organs containing cobalt destined for excretion, only the liver showed consistent accumulation. After 20 hours the livers contained about 2% of the administered dose, after 3 to 15 days about 0.1% was found and only infrequently was any labeled cobalt found in the liver 20 to 30 days after administration. In these experiments the level of administered cobalt ranged from 2 to 30  $\gamma$  and the difference in diet had no apparent effect.

*Tissue Distribution in Cattle:* Cobalt metabolism in the ruminant is of utmost importance from both the practical and fundamental standpoint. For this work, range cattle, in poor condition, were brought to

TABLE I

*Excretion of Labeled Cobalt by the Rat, in Percent of Administered Dose*

Diet	Dose ( $\gamma$ )	No. of animals	Feces	Urine
Mineral-deficient	26.4	4	87.3 $\pm$ 4.9 <sup>1</sup>	9.5 $\pm$ 0.8
Mineral-deficient	2.2	7	81.8 $\pm$ 7.2	7.0 $\pm$ 2.0
Stock	2.2	6	82.7 $\pm$ 9.5	8.0 $\pm$ 2.6
Stock	0.02	2	54.1 $\pm$ 0.7	12.7 $\pm$ 3.6

\* The mean value  $\pm$  the mean deviation.

Gainesville from an area in Florida considered deficient in some of the essential minerals. These animals were then fed on a ration consisting of redtop hay harvested from a low cobalt area, corn grown on a low cobalt area, a protein supplement of dried skim milk, and a phosphorus supplement of tricalcium phosphate. Analyses showed that this feed contained less than 0.01 p.p.m. cobalt. For about 6 months, under these conditions, most of the animals remained in poor condition and gave an appearance of emaciation, rough hair coat, and extreme weakness. The following animals were used in the work reported here:

Steer No. 1, about 2 years old, weight 250 lbs.

Steer No. 2, about 2 years old, weight 230 lbs.

Heifer No. 3, about 1½ years old, weight 195 lbs.

Cow No. 6, about 5 years old, weight 300 lbs.

The following procedure for the slaughter of the animals and handling of tissues has been found most convenient: The animal is stunned by a blow on the head and then suspended head down for complete bleeding from the jugular veins and carotid arteries, which

TABLE II

*Distribution of Labeled Cobalt in Cattle 10 Days after Administration, in  $\gamma$  per 100 g. Fresh Weight*

Tissue	Steer No. 1 (1330 $\gamma$ given orally)	Heifer No. 3 (2400 $\gamma$ injected into jugular vein)
Pituitary	†	*
Thyroid	†	0.64
Thymus	†	0.087
Adrenals	†	3.1
Reproductive organs	†	0.38
Brain	†	0.20
Eye	†	0.11
Lymph glands	0.033	0.76
Hemolymph glands	—	†
Salivary glands	—	0.36
Heart	†	0.34
Blood	†	0.32
Aorta	†	0.44
Lung	†	0.93
Trachea	†	0.62
Kidney	†	1.4
Kidney fat	0.051	—
Bladder	†	0.37
Urine (not excreted)	—	1.2
Tongue	†	0.11
Esophagus	†	0.64
Abomasum	0.013	0.23
Abomasum contents	†	0.13
Reticulum	—	0.24
Reticulum contents	—	*
Omasum	—	0.20
Omasum contents	—	*
Rumen	†	0.076
Rumen contents	†	*
Small intestine	0.030	0.49
Small intestine contents	0.047	0.18
Large intestine	†	0.21
Large intestine contents	†	0.12
Pancreas	†	*
Spleen	0.11	0.54
Liver	0.27	2.2
Gall Bladder	†	0.57
Bile	*	0.021
Tenderloin muscle	†	0.036
Gastrocnemius muscle	*	0.099
Ligament (nuchal)	†	0.39
Cartilage (costal)	†	0.39
Bone (femur)	†	0.088
Red bone marrow (ribs)	†	0.78
White bone marrow (long bones)	†	0.21
Teeth	†	0.050

\* Radioactivity measurements made but amount in sample less than 0.01  $\gamma$ .

are severed. A sample of blood for the activity measurements is obtained during the bleeding. The tissues and organs are then removed, care being taken to avoid contamination. The tissues and organs are weighed immediately upon removal and, when desirable, representative portions are taken for the activity measurements; when there is any evidence of contamination, the samples are thoroughly washed. In the case of the abomasum, reticulum, omasum, rumen, small intestine, large intestine, bladder and gall bladder, the contents are removed and weighed separately; the tissue samples from each of these organs are washed thoroughly to remove all traces of the contents.

Table II shows the effect of method of administration on the tissue distribution; the cobalt was given to one animal orally and to the other by injection into the jugular vein. In the former case the picture was much the same as in the rat, with very little cobalt being retained in the tissues and the greatest accumulation occurring in the liver which contained 0.25% of the administered dose. However, when the cobalt was injected there was a general distribution throughout the tissues, yet less than 5% of the administered dose was retained by the animal with 1% being found in the liver. Marked accumulation occurred in the adrenals.

It is interesting to note that, when the cobalt was injected, significant amounts appeared in the abomasum contents while none was found in the contents of the reticulum, omasum or rumen. This observation is confirmed by the data in Table III, obtained from other

TABLE III

*Appearance of Injected Labeled Cobalt in the Ruminant Stomach and Contents, in  $\gamma$  per 100 g. Fresh Weight*

	Cow No. 6, 260 $\gamma$ dose sacrificed after 2 hrs.	Steer No. 2, 1330 $\gamma$ dose sacrificed after 2½ hrs.	Heifer No. 3, 2400 $\gamma$ dose sacrificed after 10 days
Abomasum	0.31	1.3	0.23
Abomasum contents	0.39	0.038	0.13
Reticulum	0.19	0.60	0.24
Reticulum contents	0.00091	**	**
Omasum	0.12	0.60	0.20
Omasum contents	*	**	**
Rumen	0.16	0.70	0.076
Rumen contents	0.0038	**	**

\* Radioactivity measurements made but amount in sample less than 0.001  $\gamma$ .

\*\* Amount in sample less than 0.01  $\gamma$ .

animals which received cobalt injections and were sacrificed after 2 and 24 hours. Note also that the abomasum was one of the few tissues containing any labeled cobalt after oral administration. The cobalt in the rumen contents of cow No. 6 probably reached there via the saliva, as other work, as yet unpublished, has shown that small amounts of injected cobalt are found in the saliva; this animal received no food or water after the injection so that there was no dilution of the rumen contents. Appreciable amounts of injected cobalt have been found to appear quickly in the bile and it is possible, although unlikely, that regurgitation of the bile might account for the activity in the abomasum contents. Whether or not the cobalt is actually secreted by the abomasum it is evident that significant amounts are found in the abomasum contents and that very little injected cobalt finds its way into the contents of the other stomach compartments. It has been reported (2) that cobalt injection is apparently not as effective as oral administration in alleviating deficiency symptoms and suggested that the beneficial action of cobalt is localized in the rumen. This theory is not in disagreement with the data presented here.

The writers wish to express appreciation to Drs. Robley D. Evans and John W. Irvine, Jr. for their cooperation in supplying the radioactive materials and to Dr. R. S. Glasscock for his assistance with the livestock phase of this program.

#### SUMMARY

1. Procedures for the use of radioactive cobalt in biological studies are presented. With the best preparation available so far, the initial measurements were sensitive to 0.0006  $\gamma$  cobalt and samples as large as 500 g. have been used.

2. When 2 to 30  $\gamma$  labeled cobalt were administered orally to rats on a stock or low mineral diet about 80% of the dose was eliminated in the feces, 10% rapidly eliminated in the urine, and very little retained in the tissues, with only the liver consistently accumulating significant amounts.

3. When cobalt was administered orally to a steer, very little was found in the tissues 10 days later; the largest amount was found in the liver which contained 0.25% of the administered dose. When the cobalt was injected into the jugular vein of a heifer there was a general distribution throughout the tissues; after 10 days about 5% of the administered dose was retained by the animal with 1% being found in the liver.

4. Significant amounts of injected labeled cobalt appeared in the abomasum contents, while none was found in the rumen, omasum and reticulum contents.

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# The Protein-Formaldehyde Reaction

## The Question of Methylene Bridges and the Unreactivity of Benzoyl-d(-)-Alanine toward Formaldehyde \*

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### INTRODUCTION

Studies on the aldehyde binding of amino acids and proteins (2, 6) and X-ray studies of the structure of proteins (1) and aldehyde-treated amino acids (16) and proteins (4, 18) have done much to clarify the general problem of the protein-aldehyde reaction.

From their extensive X-ray studies Astbury and co-workers concluded that proteins consist of parallel peptide chains about 4.5 Å apart, the individual amino acid residues each having a length of about 3.5 Å along the chains. The layer of parallel peptide chains so formed is connected with other similarly constructed layers by means of the side-chains of the amino acids. Inasmuch as the layer-layer distance is about 10 Å, this means that the side-chains of only arginine and lysine and those of glutamic and hydroxyglutamic acids are of sufficient length to be employed in the layer-layer linkage. Assuming compound formation between the side-chain  $\text{—NH}_2$  and  $\text{—COOH}$  groups of these acids, a calculated distance of 10.4 Å results while a distance of 9.9 Å results if the linkage is by means of hydrogen bond (2). Either of these types of layer-layer linkage yields distances within the error of the X-ray measurements. Probably the most general means yet advanced of holding together the parallel peptide chains in the layer level is by hydrogen bonds, forming a  $\text{—COHN—}$  linkage between peptide chains and having a total bond distance of 4.65 Å (13). In certain proteins (wool) the parallel peptide chains may be held together by side-chain groups of two cysteine residues, the  $\text{—SH}$  groups of which have been oxidized to a cystine  $\text{—S—S—}$  linkage, which yields a distance of 4.66 Å between parallel peptide chains. Recently the stretch of wool has been increased by chemical reduction of  $\text{—S—S—}$  groups and reactions introducing  $\text{—CH}_2$  groups between the two sulfur atoms (8). As we have pointed out before, in protein-aldehyde

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plastics the side-chains of hydroxyamino acids may react with aldehyde to give an  $\text{—O—CH}_2\text{—O—}$  linkage, bonding together peptide chains with a distance of 4.67 Å.

Küntzel (11) was unable to account for all the aldehyde bound during the tanning of leather as reacting with  $\text{—NH}_2$  groups and proposed that the imino group of the peptide (CONH) linkage might possibly react with aldehyde to give methylene bridges between peptide chains. This hypothesis of methylene bridges has been mentioned frequently in the literature (7, 9, 17), and many seem to assume that it rests on proof. The purpose of this paper is to point out that the methylene bridge ( $\text{—CH}_2\text{—}$ ) can bridge only a distance of 2.40 Å between nitrogen atoms or 2.52 Å between carbon atoms or 2.46 Å between nitrogen and carbon atoms of parallel peptide chains and any of these are quite impossible if we accept the experimental evidence of X-ray data which show the distance between peptide chains to be 4.5 Å.

To throw direct experimental evidence on the question of whether the imino group of the  $\text{—CONH}$  linkage reacts with formaldehyde, we have employed benzoylalanine. Whatever the substance employed, it should be as simple as possible in structure and carry the  $\text{—CONH}$  group. In the strictest sense of the word, benzoylalanine is not a peptide although it contains the  $\text{—CONH}$  group. It has the experimental advantage over a true peptide, however, of having no terminal  $\text{—NH}_2$  group (such as glycylalanine) which would bind aldehyde and confuse the interpretation of results, and the benzoyl portion has the further advantage of being the acyl group of a strong acid and analogous in strength to that of a methylolamino acid which has lost any basic properties the parent amino acid may have had originally.

In reacting simple amino acids with formaldehyde, we have shown with aspartic and glutamic acids and with asparagine that the side-chain group attached to the  $\alpha$ -carbon atom, although itself not reacting with aldehyde, regulates the equilibrium constant of the reaction with the second mole of aldehyde; the longer the side-chain the greater the concentration of aldehyde required to complete the reaction. In our experiment a short side-chain is desired, therefore, on the  $\alpha$ -carbon atom, to facilitate completion of the reaction with a minimum of aldehyde. Clearly the side-chain cannot be shorter than a  $\text{—CH}_3$  group (alanine) and maintain an optically active amino acid as is required by our experimental method. We have employed the benzoyl derivative of *d*-alanine instead of the naturally occurring *l*-alanine

because we had a supply of the derivative of the *d*-acid, and its reaction toward aldehyde should be identical with that of the naturally occurring *l*-acid.

Neuberger (14) was unable to condense formaldehyde with *N*-acetyl- or *N*-benzoyl-*l*(-)-histidine in a twenty-four hour period when used in molar proportions. We have employed a range of aldehyde concentrations reaching to sixteen-fold and our method permits the use of unlimited time for equilibrium to be established between the reactants.

It is well established that formaldehyde reacts at both imino groups of diketopiperazines (3) giving methylol derivatives, but ketopiperazines have many properties not common to chain compounds. In the work reported here it has been found that there is no indication of reaction between formaldehyde and benzoyl-*d*(-)-alanine solutions containing an equivalent of sodium hydroxide.

#### PREPARATION OF MATERIALS

*Benzoyl-d(-)-alanine.* Benzoyl-*dl*-alanine was prepared by benzoylating a commercial sample of *dl*-alanine by the method of Fischer (5) and after precipitation with acid, filtration, drying and extraction of free benzoic acid with petroleum ether, the benzoyl-*dl*-alanine was recrystallized from water. Separation of benzoyl-*l*(+)-alanine as the very insoluble crystalline strychnine salt (15) and its removal from the very soluble antipode left benzoyl-*d*(-)-alanine in solution, which after conversion to the sparingly soluble crystalline brucine salt, followed by removal of brucine, gave a good yield of benzoyl-*d*(-)-alanine, which last was recrystallized twice from hot water as gable-shaped prisms and dried over phosphorous pentoxide. Melting point 148° (corr.). The rotation of a solution containing 1.5000 g. benzoyl-*d*(-)-alanine and 7.8 ml. of *N* potassium hydroxide solution (1 equivalent) and water to a total weight of 15.147 g. (sp. gr. 1.0427) was - 7.70° (2 dm).  $[\alpha]_D^{20} = - 37.25^\circ$ . Fischer gives  $[\alpha]_D^{20} = - 37.3^\circ$ .

*Formaldehyde.* A very pure concentrated formaldehyde solution was brought to exactly pH 7.0, measured against the glass electrode, by addition of sodium hydroxide solution and the aldehyde content of this stock solution determined by the sodium bisulfite method of Kleber (10).

#### EXPERIMENTAL

Exactly 24.137 g. of dry benzoyl-*d*(-)-alanine (0.125 mole) were weighed out, about 30 ml. of water added, and 67.3 ml. of a 1.860 *N* sodium hydroxide solution (1 equivalent) added and the solution made up to 125 ml. This made a stock solution in which 10 ml. contained 0.01 mole of the sodium salt of benzoyl-*d*(-)-alanine. Ten ml. portions of this stock solution were pipetted into a series of 25-ml. flasks and various amounts of the stock formaldehyde solution added to each and the volume of each solution made up to 25 ml. Part of each of these solutions was trans-



ferred to a 2 dm. polarizing tube and the remainder of the solution and the polarizing tube were placed in the constant temperature bath at 20°C. and read periodically. A constant value for the angular rotation was noted immediately, which indicated that no reaction was taking place between benzoyl-*d*(-)-alanine and formaldehyde. To make sure that an extremely slow reaction was not taking place, the final readings were made after sixty-five days at 20°C. The sodium arc was employed as a light source.

The hydrogen-ion concentration of each solution was measured with the glass electrode. These data, together with the readings of the angular rotation, are recorded in Table I and the angular rotation is shown graphically in Fig. 1.

TABLE I

*Data for Formaldehyde and Benzoyl-d(-)-alanine at 20°C.*

0.01 Mole benzoyl-*d*(-)-alanine and one equivalent of sodium hydroxide per 25 ml. solution

Soln. No.	Moles Formaldehyde	Angular Rotation degrees (2 dm.)	Conc. H Ion
1	0	-5.86	$1.5 \times 10^{-11}$
2	0.005	5.87	4.0
3	.010	5.88	8.0
4	.020	5.89	$1.6 \times 10^{-10}$
5	.03	5.91	2.5
6	.04	5.92	3.3
7	.06	5.96	4.5
8	.08	6.00	6.8
9	.10	6.03	7.5
10	.12	6.09	8.0
11	.14	6.15	$1.1 \times 10^{-9}$
12	.16	6.21	1.3

## DISCUSSION

From the optical rotation (Fig. 1) it is clear that there is no indication of a reaction between benzoyl-*d*(-)-alanine and formaldehyde under the conditions employed in our experiments. An equilibrium reaction between benzoyl-*d*(-)-alanine and formaldehyde would require an experimental curve concave to the *x*-axis and terminating in a constant rotation at high aldehyde concentration, whereas the rotation actually becomes continuously slightly more negative as the aldehyde content is increased. This small change in rotation is ascribed to the effect of change of solvent (some sixteen-fold change in concentration of aldehyde) and is a common phenomenon experienced with solvents having polar properties (12). Further it may be said that the

benzoyl-*d*(-)-alanine was practically all recovered unchanged on acidifying the experimental solutions.

In previous experiments on the binding of formaldehyde by amino acids (2, 6), we have found that one mole of aldehyde was rather firmly bound per mole of amino acid and that on adding more aldehyde, a second mole of aldehyde was bound reversibly. The 1:1 mole compounds seems to be a methylol derivative but we have been unable to

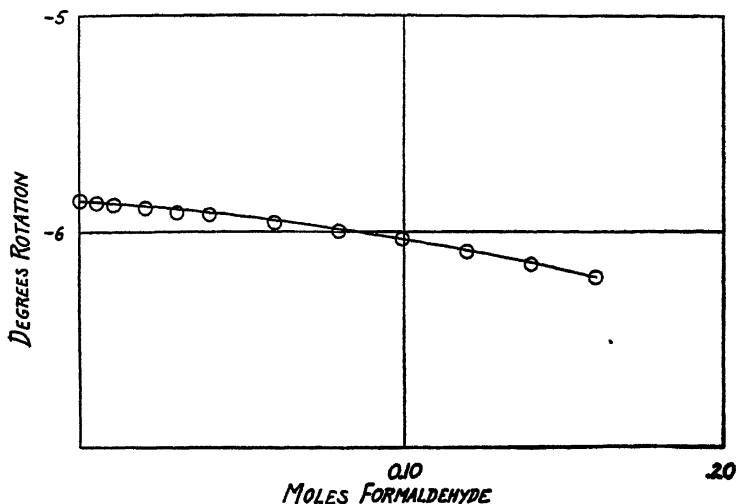


FIG. 1

Effect of Added Formaldehyde on Angular Rotation (2 dm.) of 0.01 Mole of Benzoyl-*d*(-)-Alanine Containing one Equivalent of Sodium Hydroxide in 25 ml. of Solution

say whether the second mole of aldehyde formed a dimethylol compound or whether it reacted with the first methylol group forming a compound of the type  $\text{—CH}_2\text{OCH}_2\text{OH}$ . We have indicated that the latter was probable, because of the unstable character of the 1:2 mole compound. The results with benzoyl-*d*(-)-alanine indicate that we can be fairly sure that the imino part of the peptide linkage does not react with aldehyde, so that methylene linkages between peptide chains are impossible as demanded by X-ray data.

Acknowledgment is made for the assistance of Dr. F. E. Lovelace in reading some of the rotations.

## SUMMARY

1. Calculations of bond distances show that methylene groups cannot be expected to bridge the distance found by X-ray diffraction methods between parallel peptide chains in proteins.

2. Experiments carried out with benzoyl-*d*(-)-alanine have shown no indication of reaction between the imino group of the —CONH linkage and formaldehyde, which would be a necessary prelude to the formation of methylene bridges between peptide chains.

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# The Effect of Estrogens on the Succinoxidase System of Liver and Pituitary Tissues \*

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## INTRODUCTION

It is well established that natural as well as synthetic estrogens are important in controlling the function of reproductive tissues, and they are also known to affect the metabolism and function of other tissues not directly concerned with reproduction. It seems probable that these changes depend primarily on the part played by the estrogens in the enzymatic processes concerned with cellular function. As a means of obtaining information on these cellular processes a study was instigated to determine the effect of the estrogens on the activity of certain respiratory enzymes. This report presents results obtained from experiments designed to determine the effect of estrogens on the succinoxidase system of liver and pituitary tissues. However, because of the insolubility of the natural estrogens under the conditions used, the report is concerned mainly with the effect of synthetic estrogens.

## EXPERIMENTAL

The liver and pituitary tissues were obtained from adult rats of the Sprague-Dawley strain, and pituitary tissue was obtained also from immature rats of the same strain which had been used for assay of gonadotropic extracts.

The animals were killed by decapitation. The samples of liver and pituitary tissues were removed immediately, weighed and placed in 0.5 ml. of cold glass-distilled water which was in an homogenizing tube. The "homogenization" method of Potter and Elvehjem (1) was used in preparing the tissue homogenates. Sufficient water was added to the homogenized tissue to make a 5% homogenate.

The proper amounts of the homogenates (usually 0.2 ml. of liver and 0.3 ml. of

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pituitary) were placed in the Warburg flasks with fortified substrate (2, 3). The fortified substrate solution consisted of 1 ml. of 0.1 *M* phosphate buffer of pH 7.4, 0.3 ml. of 0.5 *M* sodium succinate, 0.3 ml. of 0.004 *M*  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.3 ml. of 0.004 *M*  $\text{AlCl}_3$ , and 0.2 ml. of  $3 \times 10^{-4}$  *M* cytochrome *c*. This concentration of cytochrome *c* was previously shown (4) to be sufficient to take care of the dilution effect (3). In addition, the proper amounts of 0.001 *M* solutions of the compounds tested for inhibitor activity were added to the flasks with the tissue and the substrate. Sufficient water was also added to make a total volume of 3 ml.; 0.1 ml. of 20% potassium hydroxide was used in the center wells. The experiments were carried out in duplicate flasks containing a constant amount of homogenate, as it had been shown previously that, under the above conditions, the oxygen uptake is directly proportional to the amount of tissue homogenate used (4).

Approximately 20 minutes elapsed from the time the tissue was removed from the animal until the flasks were placed in the bath of the Warburg apparatus at 38°C. A period of 10 minutes was allowed for the contents of the flasks to come to the temperature of the bath and the oxygen uptake was obtained by taking readings at 10 minute intervals. The results are reported in terms of  $\text{QO}_2$ ; i.e., the mm.<sup>3</sup> of oxygen taken up per mg. of dry tissue per hour. The  $\text{QO}_2$  values reported are averages based usually on the first four 10 minute periods.

The cytochrome *c* was made from beef heart muscle according to the method of Keilin and Hartree (5) except that the final product was dialyzed against glass-distilled water instead of 1% sodium chloride solution. A 0.5 *M* solution of Eastman sodium succinate was adjusted to pH 7.4 and used as substrate.

A 0.114 *M* solution of Merck ascorbic acid was employed as substrate for cytochrome oxidase (6). This substrate was used to determine whether the inhibitor acted on cytochrome oxidase. Otherwise the medium was the same as that for succinic dehydrogenase except 0.3 ml. of  $9 \times 10^{-4}$  *M* cytochrome *c* was used with 0.2 ml. of 2% liver homogenate.

To test in another way whether the inhibitor acted on cytochrome oxidase, the cytochrome *c* was replaced by brilliant cresyl blue (7). Five-tenths ml. of a 0.5% solution of the dye was utilized.

Diethylstilbestrol, hexestrol and dienestrol\* (4,4'-dihydroxydiethyldenediphenylethane) were each dissolved in dilute sodium hydroxide in order to test their effectiveness as inhibitors. Solution was effected by adding 0.5 ml. of water to 2.7 mg. of each compound plus 0.04 ml. of 2 *M* NaOH after which the particles were stirred with a small glass rod until solution was complete. To neutralize a part of the NaOH, 0.01 ml. of 2 *M* HCl was added plus sufficient water to make a volume of 10 ml., which gave a concentration of 0.001 *M* estrogen. Diethylstilbestrol and hexestrol can also be dissolved by use of 0.03 ml. of the NaOH with 0.01 ml. of HCl. In fact, in the first experiments HCl was added until the compound was just soluble, but, with solutions of this kind, consistent inhibition was not obtained as the estrogen tended to precipitate when added to the fortified substrate which had a pH of 7.4.

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\* We are indebted to Abbott Laboratories, to Dr. C. W. Sondern of the White Laboratories, and to Dr. A. L. Wilds of the Dept. of Organic Chemistry, University of Wisconsin, for samples of the various synthetic estrogens used in this work.

Consequently, as a precaution against precipitation, 0.03 or 0.04 ml. of 2 *M* NaOH was used with 0.01 ml. of 2 *M* HCl in dissolving these estrogens.

A control solution was made by mixing 0.03 ml. or 0.04 ml. of 2 *M* NaOH, depending on the amount employed in dissolving the estrogen, with 0.01 ml. of 2 *M* HCl and addition of water to make a volume of 10 ml. Three-tenths ml. of this solution was added to Warburg flasks for use as controls for the flasks that received 0.3 ml. of the 0.001 *M* aqueous estrogen solutions.

Disodium 3,4-diphenylhexane-*p-p'*-dioxyacetate, which is a water-soluble compound of low estrogenic activity, was tested. Sodium 3,4-diphenylhexane-*p*-hydroxy-*p'*-oxyacetate was also tested; 3.3 mg. of the acid was dissolved in 5 ml. of water by use of 0.01 ml. of 2 *M* NaOH. The stock solutions of these compounds were 0.001 *M* and 0.002 *M*. Appropriate controls were used.

Attempts were made to employ the same method to dissolve the natural estrogens—estrone, estradiol and estriol—in order to test their effectiveness in inhibiting the succinoxidase system. It required from 0.2 ml. to 0.4 ml. of 2 *M* NaOH to dissolve the proper amounts of these hormones to make 10 ml. of 0.001 *M* solutions. As much as 0.1 ml. of 2 *M* HCl could be added without causing these substances to precipitate. However, when these solutions were added to the buffer substrate at pH 7.4 or even at pH 8, precipitation occurred, so that the results obtained did not indicate whether the natural estrogens are effective inhibitors. However, an impure sample of sodium estrone sulphate<sup>+</sup> was obtained and used as it is water-soluble. The stock solution of this compound was 0.002 *M*. The results obtained with this sample of sodium estrone sulphate are only indicative, which makes further study of the effect of the natural estrogens on the succinoxidase system desirable.

## RESULTS AND DISCUSSION

The data in Table I demonstrate that diethylstilbestrol, hexestrol and dienestrol are effective inhibitors of the succinoxidase system of rat liver. Diethylstilbestrol and hexestrol obtained from two different sources were found to be about equally effective. The results of Table II show that diethylstilbestrol inhibits the succinoxidase system of pituitary tissue from immature and adult rats. This would be expected since, as was indicated above, this estrogen inhibited succinoxidase of rat liver. Although the degree of inhibition is greater in the case of pituitary tissue from young animals, this difference is probably not significant as the  $Q_{O_2}$  values obtained in the different experiments with tissue from adult animals were variable. In addition only one run was made with pituitaries from young rats. Malonate was also shown to be effective in the inhibition of the system in pituitary tissue.

The question arose as to whether the degree of inhibition of the succinoxidase system by diethylstilbestrol was proportional to the

<sup>+</sup> Supplied by Dr. J. M. Scott of Ayerst, McKenna and Harrison.

TABLE I

*Effect of Estrogens on the Succinoxidase System of Rat Liver Tissue*

Age of rat	No of runs	Q <sub>O<sub>2</sub></sub>			
		No inhibitor	Final molarity of inhibitor (10 <sup>-4</sup> )		
			1.0	2.0	4.0
Adult	9	82.9(65.6-106.3)	Diethylstilbestrol		
			18.7(10.4-28.4)	—	—
Adult	5	76.6(57.3-86.4)	Hexestrol		
			18.0(7.0-29.2)	—	—
Adult	3	87.1(67.4-106.3)	Dienestrol		
			19.8(10.5-30.7)	—	—
Adult	5	81.7(63.3-99.8)	Sodium 3,4-diphenylhexane- <i>p</i> -hydroxy- <i>p'</i> -oxyacetate		
			76.8(54.9-88.8)	68.5(52.7-92.7)	47.5(51.1-43.9)
Adult	4	86.1(78.7-89.8)	Disodium 3,4-diphenylhexane- <i>p,p'</i> -dioxyacetate		
			87.6(86.0-88.8)	85.3(78.1-88.9)	84.0(76.4-92.0)
Adult	2	78.9(78.9-79.0)	Sodium estrone sulphate		
			69.2(68.9-69.6)	64.8(60.9-66.9)	50.2(48.1-52.1)

concentration of the estrogen. To answer this question experiments were made using five different concentrations of the hormone varying from 0.2 to  $2 \times 10^{-4}$  *M* with a constant amount of liver homogenate. The results are given in Table III, and these data show that the inhibition increases with increasing concentrations of the hormone. The highest concentration used,  $2 \times 10^{-4}$  *M*, gave complete inhibition in two experiments and almost complete in the third.

TABLE II

*Effect of Diethylstilbestrol and Malonate on the Succinoxidase System of Rat Pituitary Tissue*

Age of rat	No. of runs	Q <sub>O<sub>2</sub></sub>		
		No inhibitor control	Diethylstilbestrol Final molarity of inhibitor (10 <sup>-4</sup> )	
			1.0	5.0
26 da.	1	24.0	4.6	—
Adult	7	23.7(19.5-27.3)	10.6(4.7-18.3)	16.0(13.6-18.8)
			Disodium 3,4-diphenylhexane- <i>p,p'</i> -dioxyacetate	
Adult	1	23.5	22.5	—

TABLE III

*Inhibition of the Succinoxidase System of Rat Liver by Different Concentrations of Diethylstilbestrol*

Experiment No.	Control	Q <sub>O<sub>2</sub></sub>				
		0.2	Final molarity of inhibitor (10 <sup>-4</sup> )	0.5	1.0	2.0
1	88.0	71.3	—	40.6	20.8	0.0
2	80.4	72.9	63.3	52.0	28.3	0.0
3	77.1	—	—	38.4	23.8	2.2
Average	81.8	72.1	63.3	43.6	24.3	0.7

The effectiveness of diethylstilbestrol as compared with malonate in the inhibition of the succinoxidase system was studied by using 10<sup>-4</sup> *M* concentrations of these compounds. The results of experiments 1 and 2 are given in Table IV. These data show that diethylstilbestrol is much more effective than malonate. The concentration of malonate in experiment 3 was  $4 \times 10^{-4}$  *M* which was four times that of diethyl-

TABLE IV

*Comparison of the Effectiveness of Malonate and Diethylstilbestrol in the Inhibition of the Succinoxidase System of Rat Liver*

Experiment No.	No inhibitor control	Q <sub>O<sub>2</sub></sub>				
		Diethylstilbestrol	Final molarity of inhibitor (10 <sup>-4</sup> )			
		1.0	1.0	4.0	5.0	10.0
1	85.4	20.8	80.7	—	50.3	25.7
2	86.7	17.1	74.9	—	—	—
3	60.1	6.1	—	38.0	—	—

stilbestrol. Under these conditions the Q<sub>O<sub>2</sub></sub> was decreased from the control value of 60.1 to 6.1 by the estrogen as compared with 38.0 for malonate.

It was first considered that the mechanism of the inhibition by these estrogens consisted of competition with the substrate succinate for the enzyme succinic dehydrogenase, as has been shown to be the case for inhibition by malonate. However, since it was found that diethylstilbestrol was much more effective than malonate, and that in a concentration of  $2 \times 10^{-4}$  *M* it inhibited completely, the question arose as to whether the inhibitory effect might not be exerted on



cytochrome oxidase instead of succinic dehydrogenase. A suggestion that this might be the case was the fact that the cytochrome c in the flasks with the diethylstilbestrol was observed to be in the reduced state immediately after conclusion of the experiments.

To determine whether the cytochrome oxidase was inhibited, brilliant cresyl blue was used in place of cytochrome c. Weil-Malherbe (7) showed that the succinoxidase system would function without cytochrome c in the presence of this dye although not to the same extent as with cytochrome c. The fact that the dye is autoxidizable in the presence of oxygen permits the system to function without the action of cytochrome oxidase. Thus, in a system in which cytochrome c is replaced by the dye, if the inhibition by diethylstilbestrol

TABLE V

*Effect of Brilliant Cresyl Blue on the Inhibition of the Succinoxidase System of Liver by Diethylstilbestrol*

Sod. succ. 0.5 M	Cytochrome c $3 \times 10^{-4}$ M	Brilliant cresyl blue 0.5%	Diethylstil- bestrol $10^{-3}$ M*	Qo <sub>2</sub> Experiments		
				I	II	III
ml.	ml.	ml.	ml.			
0.3	0.2	—	—	77.5	53.7	68.0
0.3	0.2	—	0.3	5.9	12.7	27.3
0.3	—	0.5	—	37.0	32.5	42.1
0.3	—	0.5	0.3	31.3	30.2	38.8

\* Final concentration was  $10^{-4}$  M when 0.3 ml. was used.

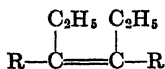
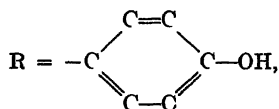
is affected by combination with cytochrome oxidase, the oxygen uptake should be approximately the same both in the absence and presence of inhibitor. The details of the experiments are given in Table V. The results show that diethylstilbestrol inhibited when cytochrome c was used but when it was replaced by brilliant cresyl blue there was little inhibition as, under this condition, cytochrome oxidase was not required. Thus, these results support the view that diethylstilbestrol inhibits cytochrome oxidase and not succinic dehydrogenase.

In order to test further whether the inhibitory effect of diethylstilbestrol was exerted on cytochrome oxidase, an experiment was made using ascorbic acid as substrate in the presence of cytochrome c, both with and without the estrogen. The Qo<sub>2</sub> for cytochrome oxidase without the inhibitor was 317 as compared with 153 in the presence of diethylstilbestrol. These results also show that the inhibition of

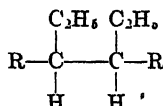
the succinoxidase system is due to the action of the diethylstilbestrol on cytochrome oxidase.

The inhibition exerted by diethylstilbestrol was as great during the first 10 minute period as during the later periods. It should be stated, however, that just prior to the first ten minute period, ten minutes were allowed for the contents of the flasks to come to the temperature of the bath during which time the inhibitor was in contact with the enzyme and the substrate.

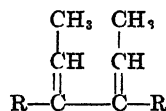
The similarity in structure of the synthetic estrogens, as shown by the following formulae, make it seem probable that the inhibitory mechanism for substances of this type is the same. The formulae are given below, where



Diethylstilbestrol

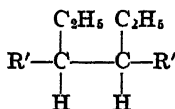
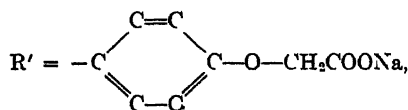
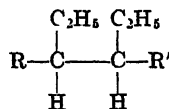


Hexestrol



Dienestrol

However, the structure of disodium 3,4-diphenylhexane-*p,p'*-dioxyacetate is similar to the others of this type except that the 4,4'-phenolic hydroxyl groups are replaced by oxyacetate groups. This compound is water-soluble but did not inhibit cytochrome oxidase. Sodium 3,4-diphenylhexane-*p*-hydroxy-*p'*-oxyacetate, which contains one free phenolic group, inhibited, but the degree of inhibition was less than that obtained with the dihydroxy compounds such as diethylstilbestrol, hexestrol and dienestrol (Table I). In the formulae for the oxyacetate compounds,

Disodium 3,4-diphenylhexane-*p,p'*-dioxyacetateSodium 3,4-diphenylhexane-*p*-hydroxy-*p'*-oxyacetate

The monoxyacetate is also less active estrogenically than the diphenolic compounds but more active than the dioxyacetate. In this connection it is of interest that estrone which has one phenolic group, when used in the form of the sulphate, inhibited to about the same degree as the monoxyacetate (Table I). Estrone is also less active estrogenically than stilbestrols having two phenolic groups such as diethylstilbestrol. These results suggest that the estrogenic activity of estrogens may be correlated with their ability to inhibit the succinoxidase system.

The correlation of the degree of inhibition with the decrease in the number of terminal phenolic groups suggests that these groups are necessary for inhibition to take place. This does not seem unreasonable as cytochrome oxidase or indophenol oxidase is known to catalyze the oxidation of certain phenolic compounds which indicates that this enzyme must have an affinity for this group. Thus, it may be that the phenolic groups of the estrogens combine with the active centers of the enzyme but are not oxidized, and as a result prevent the enzyme from performing its normal function.

There is a correlation between the inhibition of cytochrome oxidase by diethylstilbestrol and the diabetogenic effect of diethylstilbestrol in the rat reported by Ingle (8), the increase in fat produced in chickens by large doses of diethylstilbestrol (9, 10, 11) and the lipemia produced by natural estrogens (12). Other correlations are the impairment of the oxidative phase of carbohydrate metabolism in the prostate of the dog reported by Barron and Huggins (13, 14), and the regressive changes produced in prostate cancer by diethylstilbestrol and its dipropionate (15, 16). However, further exploration of other key metabolic enzyme systems is required before specificity can be attached to the inhibition of cytochrome oxidase by diethylstilbestrol.

We are grateful to Drs. Van R. Potter and P. P. Cohen for reading the manuscript.

### SUMMARY

The results presented show that diethylstilbestrol, hexestrol and dienestrol are effective inhibitors of the succinoxidase system. The effectiveness of diethylstilbestrol increased with increasing concentration until, with the proper concentration, complete inhibition was attained. The maximum effect of the hormone is exerted soon after it

is mixed with the enzyme. It was shown that the inhibition by diethylstilbestrol was effected through the cytochrome oxidase of the system.

Compounds having one phenolic group and less activity in the estrogenic sense are also less effective as inhibitors, and when both phenolic groups of the stilbestrols are replaced by oxyacetate groups little, if any, inhibition is obtained.

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# Electron Micrographs of Crystalline Plant Viruses

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## INTRODUCTION

In a recent note (1) we pointed out that the electron microscope offers a means of observing regularities in the arrangement of the elementary particles deposited from certain crystallizable plant virus preparations. The present paper shows additional electron micrographs which are useful in the elucidation of the way in which these virus particles deposit from solution to form crystals.

## EXPERIMENTAL AND DISCUSSION

Two purified plant viruses were photographed. One was the tomato bushy stunt virus which had been grown in tomato plants; the other was southern bean mosaic virus, cultivated in Bountiful bean plants. Crystallizable suspensions were obtained from infectious juice of diseased plants by differential ultracentrifugation, following procedures commonly employed in this type of purification. Electron micrographic observations, using an RCA Type EMB instrument, were carried out on properly diluted aqueous suspensions of the purified viruses.

Biological objects such as these viruses can be especially clearly seen and studied in the electron microscope if they are first "shadowed" by the oblique deposition on them of a thin metallic layer (2). The photographs in this paper are of such shadowed preparations. To make them, a micro drop of diluted virus suspension was placed on the surface of one of the collodion-covered metal screens customarily employed for electron microscopy. After standing for two to five

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minutes, as much as possible of the drop was withdrawn with a micro pipette and the residue allowed to dry in air. Such preparations were then put into the chamber of a high vacuum evaporating outfit and a known weight of metallic gold was obliquely deposited on them from a hot source. The heating filament in the chamber was so placed that objects on the collodion substrate shielded areas about five times their height from the depositing gold; the weight of gold was chosen so that its average calculated thickness over the surface of the preparation was 8 Å.

Very different types of fields were seen in electron micrographs of the bushy stunt virus. In one, the elementary particles appeared as well-defined spheres that packed closely together in a regular array. A typical region of this sort is shown in Fig. 1. In other parts of the preparation the particles, where they could be discerned at all, were ill-defined in clumps and small masses. Fig. 2 is such a field.

Most of the particles of Fig. 1 are regularly arranged in a layer one particle thick; in this sense it can be thought of as a sort of two-dimensional crystalline array. If the particles are truly spherical, or at least figures of revolution, and if they are distributed as are the spheres in either a cubic or a hexagonal close packing, they should be in contact with one another along three rows making angles of  $120^\circ$  with one another. This is true for the bushy stunt virus, within the accuracy of the measurements possible on Fig. 1 and similar photographs. The heights of the shadows suggest that the virus particles are strictly spherical but the angle of shadowing was not sufficiently well known to prove that this is exactly true. Fairly accurate determinations of particle diameter result from measurements along these contacting rows. In terms of a calibration based on the photography of a 14,400 line-per-inch grating replica, the diameter of the bushy stunt virus particle is 255 Å in Fig. 1. Measurements on other photographs, such as Fig. 3, yield values ranging from *ca.* 250 Å to *ca.* 270 Å, this variation probably being determined by the conditions under which the preparation was made. These diameters are in excellent agreement with the value measured on an earlier unshadowed photograph (3) of isolated virus particles and that derived from rates of sedimentation (4, 5) and diffusion.

Regions can be found on practically all "shadowed" preparations of the bushy stunt virus studied which give photographs like Fig. 2. The nature of the matrix of disordered material in which occasional

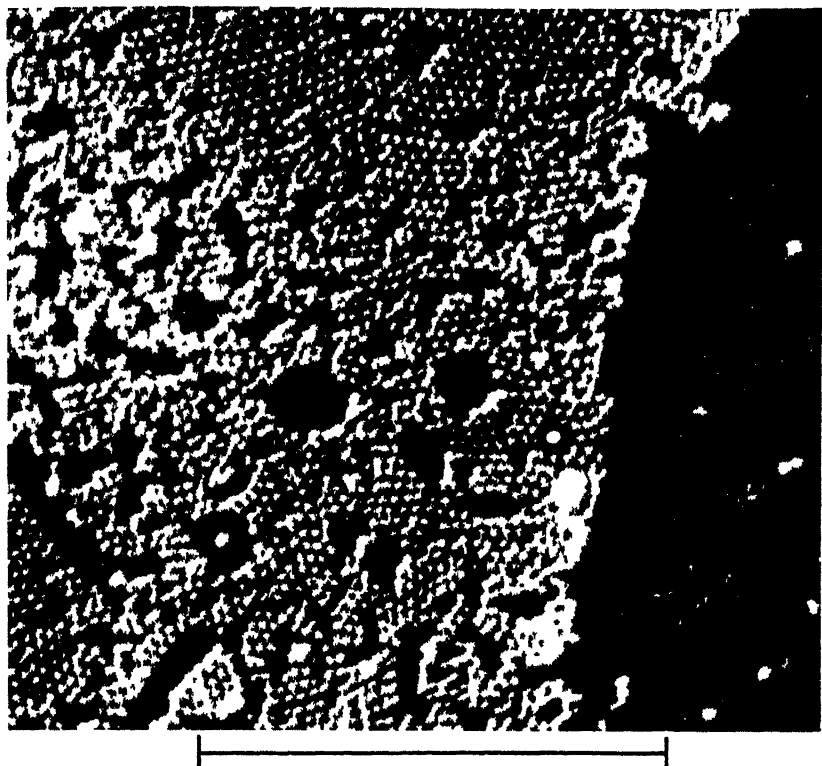


FIG. 1

A Shadowed Electron Micrograph Showing the Elementary Particles in a Purified Suspension of Bushy Stunt Virus

Their regular distribution in a single layer is evident. A few separate particles can be seen to the right. The inked line on this and the other photographs is equivalent in length to one micron on the preparation. The magnification of this picture thus is 61,000 $\times$ .

virus particles can be seen embedded is not clear. It may consist of an agglomeration of virus particles that have been severely damaged during the treatments to which the preparations were subjected.

In photographs showing greater concentrations of virus than those of Fig. 1, the particles are piled in successive layers. Such a thicker region can be seen in the center of Fig. 3. Where individual particles can be discerned, the continued regularity of their arrangement is



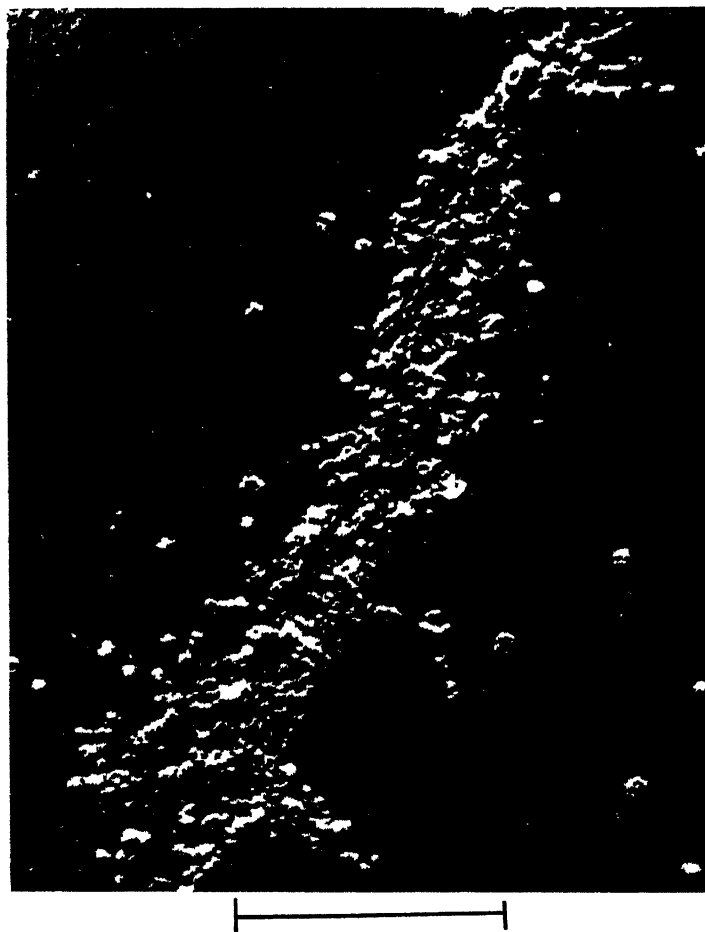


FIG. 2

A Typical Electron Micrograph Showing the Character of the Disordered Material  
Seen in Preparations of the Bushy Stunt Virus  
Magnification, *ca.* 35,000 $\times$

evident. Such a three-dimensional regularity of arrangement is by definition crystalline. Some of the photographs of southern bean mosaic virus, to be presented later, show very extensive areas of three dimensional crystallinity. Such extensive areas have not yet been observed in the bushy stunt virus preparations.

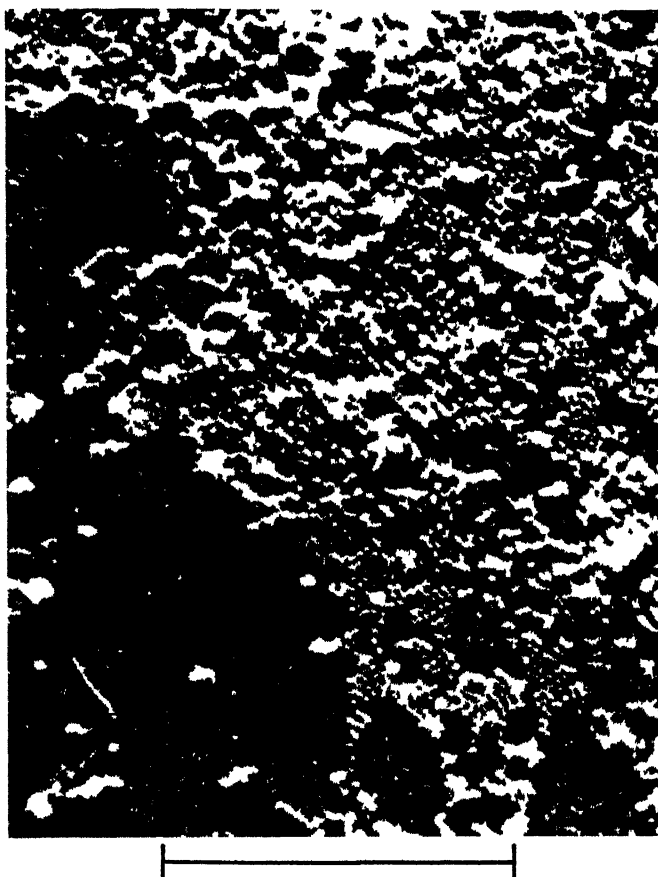


FIG. 3

A Photograph of the Bushy Stunt Virus Having its Elementary Particles  
Particles Stacked One on Top of Another  
Magnification 46,000 $\times$

In more dilute bushy stunt virus preparations the elementary particles are distributed singly and in clusters of a few individuals over the collodion substrate. A few of these separate particles and groups appear in Fig. 1; many more are evident in other photographs not presented here. It is often hard to be sure whether a chosen object is single or not, and for this reason care must be taken in drawing

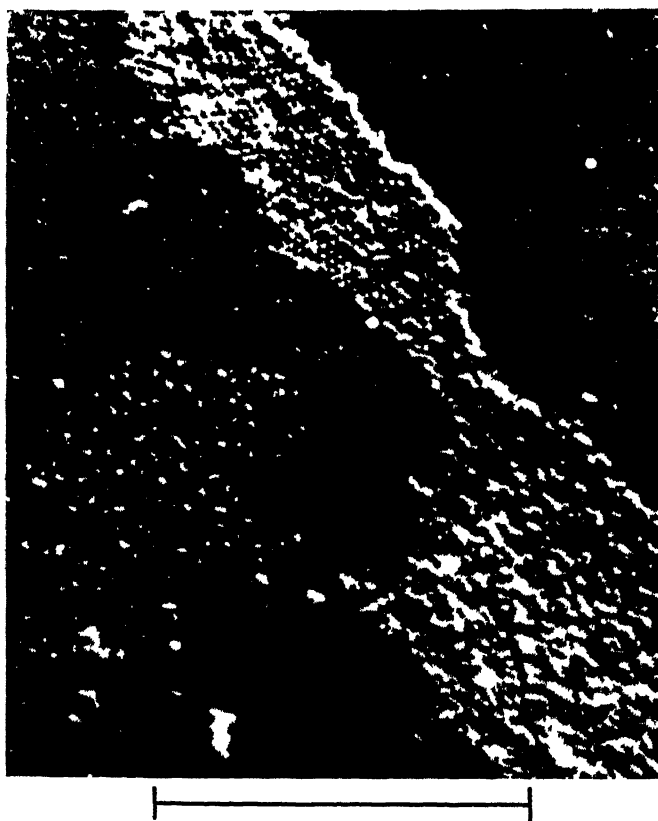


FIG. 4

Particles of the Southern Bean Mosaic Virus Exhibiting Regular  
Arrangement in a Single Layer  
Magnification 52,000 $\times$

conclusions about particle size from measurements on them and the shadows they cast.

Nevertheless, consideration of all these photographs brings out the remarkable uniformity in size of the individual particles of the bushy stunt virus. This uniformity, which has already been noted by others (3, 4, 5), is to be contrasted with the wide variation of molecular diameters observed (6), for instance, in purified solutions of the non-crystallizing hemocyanin from *Limulus polyphemus*.

Particles of southern bean mosaic virus are similar both in size and shape to those of bushy stunt virus. Regions of regular particle arrangement are far more numerous in the bean mosaic preparations, and there is a pronounced tendency for the regular layers of particles of this virus to be stacked on top of one another in a three-dimensional crystalline array. Large crystals of the bean mosaic virus (7), such as

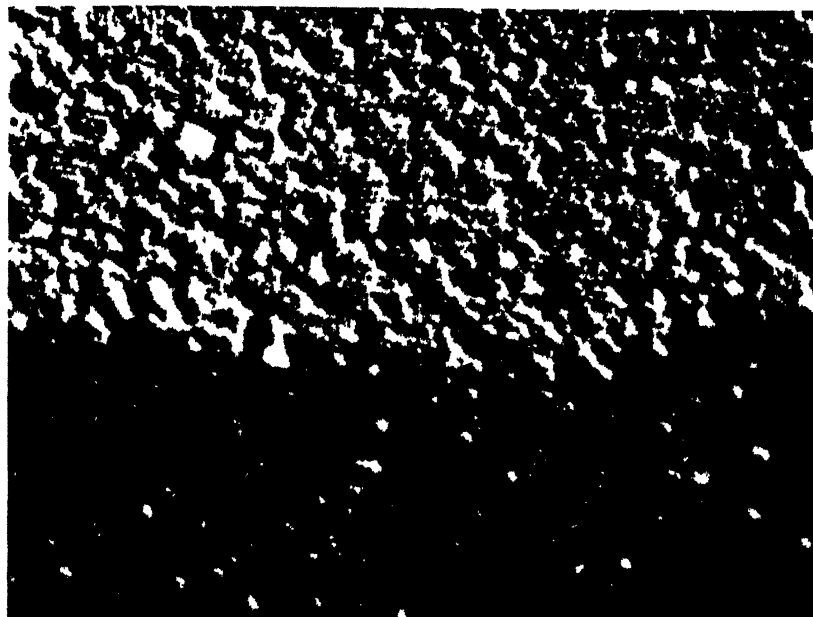


FIG. 5

Another Preparation of the Southern Bean Mosaic Virus  
At the top of the picture the distribution is in a single layer.  
Magnification 50,000 $\times$

those to be seen under the optical microscope, have less than the cubic symmetry of bushy stunt virus crystals. The data from our photographs do not show whether this lower symmetry is due to a minor asymmetry in the particles themselves, to a departure in particle arrangement from the conditions of cubic close packing, or to both factors working together.

The single-layered packing of the bean virus particles, where it occurs as in Figs. 4 and 5, is of the same general kind as the two-dimensional close packing of the bushy stunt virus. There is no evidence from these photographs that the particles of the bean virus

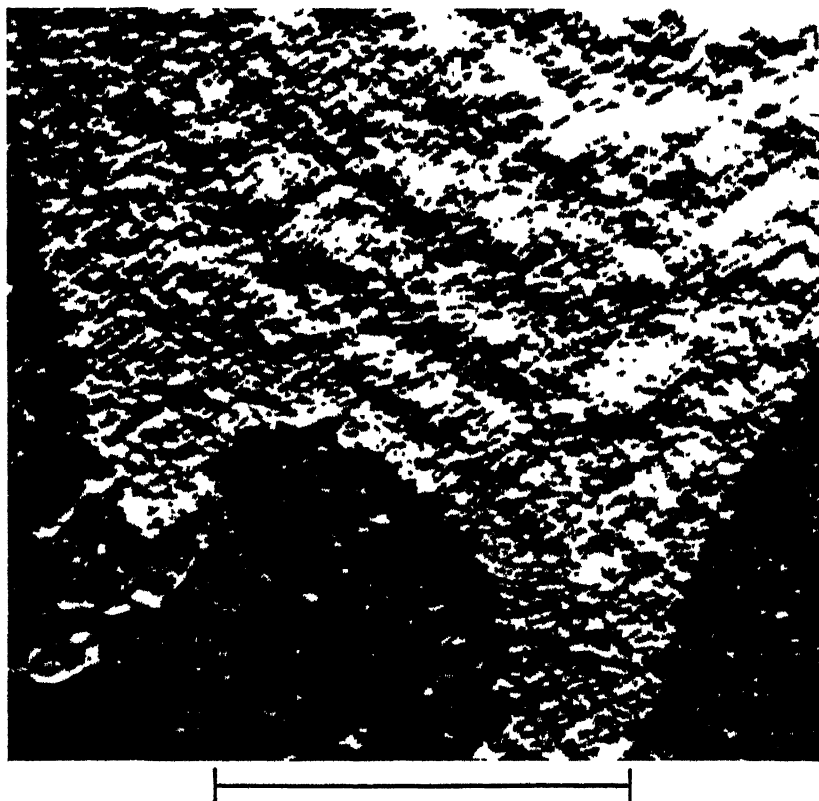


FIG. 6

An Aggregate of the Southern Bean Mosaic Virus with its Particles Beginning to Show a Regular Three-Dimensional Array  
Magnification 55,000×

are less than spherical in symmetry. They are, like the bushy stunt virus particles, very uniform in size and their measured diameter is, to a first approximation, the same as for this other virus, namely *ca.* 250 Å.

The bean mosaic virus particles associate so readily that extensive three-dimensional arrays form from sufficiently concentrated solutions. As can be seen from Fig. 6 these regions are not at first confluent

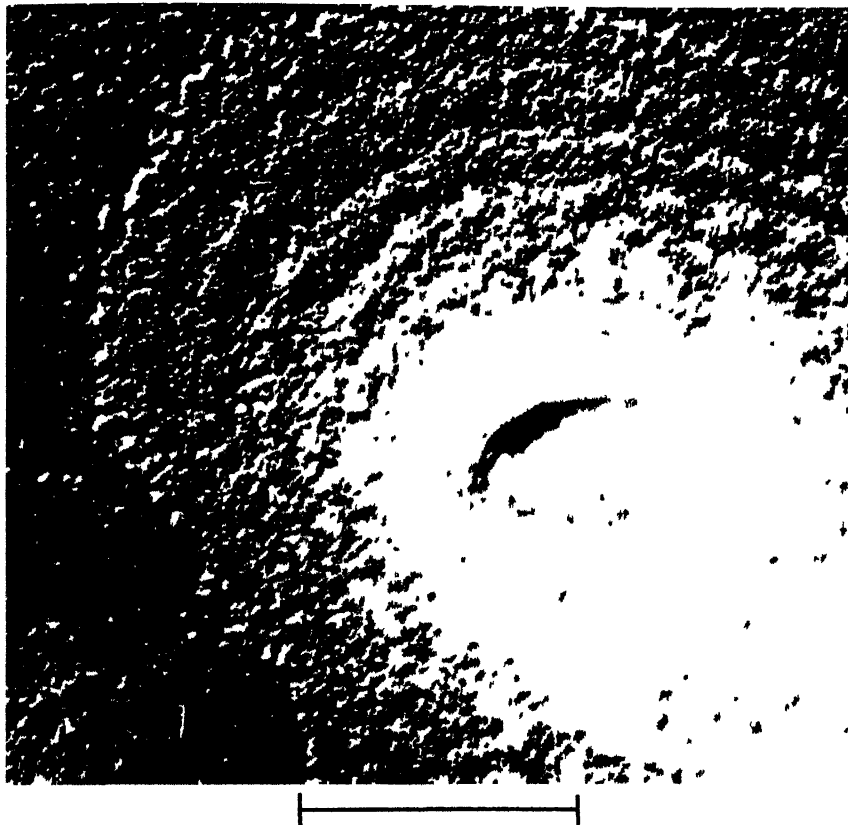


FIG. 7

A Thicker Southern Bean Mosaic Virus Preparation in Which the Islands of Three-Dimensional Regularity are Merged to Give Beaches of Increasing Thickness  
Magnification 36,000 $\times$

but consist of blocks which are similarly but not identically oriented. They can be thought of as the physical equivalents of the mosaic blocks that are assumed in the conventional picture of ideally imperfect crystals. A similar crystalline mass with the blocks merging to yield

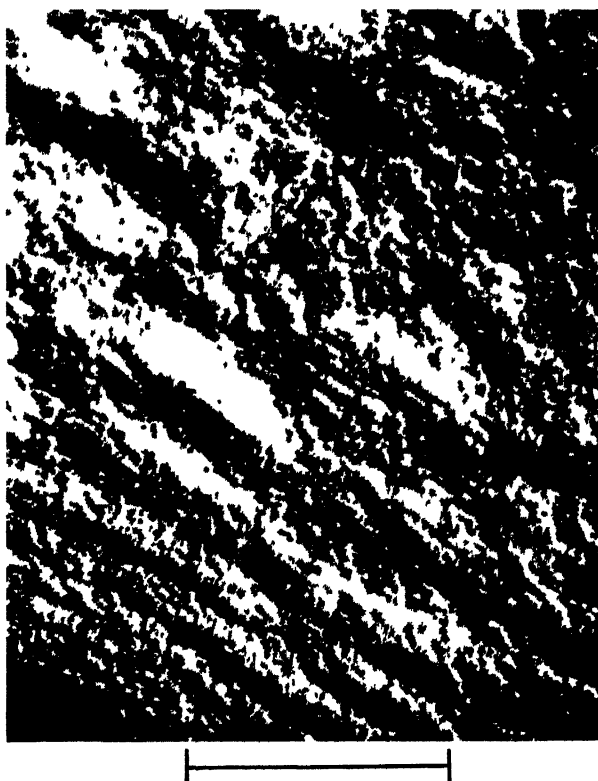


FIG. 8

A Micrograph of a Still Thicker Aggregate of Southern Bean Mosaic Virus Particles in Three-Dimensional Crystalline Array

Lines of regularly arranged particles are easily discernible although increased diffuse electron scattering makes it harder to see the individual particles of virus. Sharp crystalline edges and faces are evident at many places. An especially well-defined triangular face lies just above the step-formation at the right of the center of the photograph. Magnification 34,000 $\times$ .

beaches of increasing thickness is shown in Fig. 7. More extensive crystalline masses are evident in Fig. 8 but even here they are only rarely bounded by anything resembling smooth faces. Layers of particles cover one another to give ridges that are an exaggeration of the beaches of the two preceding pictures and that follow one another in a step-wise fashion suggestive of the Egyptian pyramids. These

steps and rows of particles seem to become more nearly parallel as the thickness increases. Around the center of the photograph many sharp edges can be seen and immediately to the right of this center is an especially flat triangular plateau which is the closest approach to a crystal face observed on any photograph. Unfortunately at this thickness diffuse electron scattering from the underlying layers limits the detail that can be photographed. A replica technique such as that described (6, 8) for minimizing the fine structure of collodion will probably be needed to give most information about particle arrangement on the surface of thick crystals.

### SUMMARY

Shadowed electron micrographs show that the elementary particles of the tomato bushy stunt and of the southern bean mosaic viruses are essentially spherical bodies of about the same size. The measured diameter of the bushy stunt particle on these photographs is 255 Å. The micrographs also provide evidence of the regular way in which the particles arrange themselves when forming crystalline aggregates.

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# Plant Growth Under Controlled Conditions. VII Sucrose Content of the Tomato Plant

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## INTRODUCTION

In a previous paper (Went 1944) it was shown that among the most easily determined carbohydrates (reducing sugars, sucrose and starch) only the sucrose concentration in a tomato plant showed consistent diurnal fluctuations. Because of the obvious relation between photosynthesis and sucrose content, the latter was followed in tomato plants grown under controlled conditions in air-conditioned greenhouses (Went 1943). This had the advantage over field observations that the observed changes were attributable to only a limited number of variables, making conclusions more binding, and over laboratory experiments that the growing conditions were near optimal.

Photosynthesis in the tomato plant has been measured previously, among others by Mitchell (1937), Christopher (1937) and Went (1944). In the latter case, photosynthesis was judged by its effect on stem elongation in plants starved for sugar. Above 1000 f.c. the leaves were found to be saturated with light. Photosynthesis was hardly greater at 26°C. than at 17°C. Old and young leaves seemed to be about equally effective in utilization of the light. The effect of light was proportional to the duration of exposure.

Sucrose content can not be used as a direct measure of photosynthesis, although it is the only carbohydrate which was found to change appreciably after illumination of the tomato plant. But it is likely that a certain proportionality exists between sucrose formed and photosynthesis and, for this reason, the sucrose content of tomato plants is of special interest.

## METHODS

Unless otherwise stated, tomato plants (San Jose Canner), grown in 4 inch pots and of the same age and size, were placed from 16:00 (4:00 P.M. Pacific War Time,

one hour ahead of sun time) until next morning 8:00 in a darkroom at 17°C. They were then placed in the greenhouse at 18°C. in full daylight, and every hour a group of 5-10 plants, selected at random, was harvested. The growing tops, with the youngest rapidly expanding leaves, were collected separately, as were the first leaves (youngest almost full grown leaves), second leaves, third leaves, etc., and the stems were cut into 10 cm. lengths and also kept separately according to position on the plant. Immediately after collection, the samples were dried in an oven with forced draft at 58°C. Sucrose was determined directly on water extracts of the dried material by increase in reduction after hydrolysis with invertase. Since the reducing sugar values did not have to be evaluated, this simplification was permissible, yet did not influence the sucrose values provided the hydrolysis was carried out with invertase (Bonner, unpublished).

The first set of determinations was made on plants collected on June 22, 1944, which happened to be an exceptionally dark and cloudy day. Only after 12:00 did the sun come through the clouds, first occasionally, but after 14:00 most clouds disappeared. This experiment will not be described in detail; a summary of results is given in Fig. 3. In most leaves the sucrose content increased more rapidly at 26.5°C. than at 18°C., and at both temperatures the sucrose values of the samples collected at 13:00 were considerably lower than those collected at 12:00.

The second set of samples was collected on September 1, 1944, a bright, sunny day. From 8:00 on, when the plants were first brought into the greenhouse, they were exposed to full sunlight. Fig. 1 combines all data of the sugar content of leaves and stems of plants kept at 18°C. during the previous night and during the exposure to light. Considering that all points on one curve are obtained by analyzing samples collected from separate sets of plants (7 each), these curves are remarkably smooth. This indicates that among the factors determining sucrose content of tomato leaves during daytime under the experimental conditions used, length of illumination exceeds all others in importance.

Another fairly consistent effect is that from the first and second leaves downward sucrose production decreases, first slowly, but abruptly in the sixth leaves. These were the lowest leaves remaining on the plants analyzed, and some of them had brown edges and were dying. This effect of age on the rate of sugar production in the leaf was also evident in the first experiment and is shown in Fig. 2. Since,

in these experiments, the sugar concentration was measured, and not the total amount formed per leaf, they do not contradict the earlier results (Went 1944) in which all leaves were found to be about equally effective in total food production.

In Fig. 1 there are only four points which are greatly off the mean expected from a smooth change in sugar content in the leaves with time. Those are the 13:00 values for the 1st leaves, and the 14:00

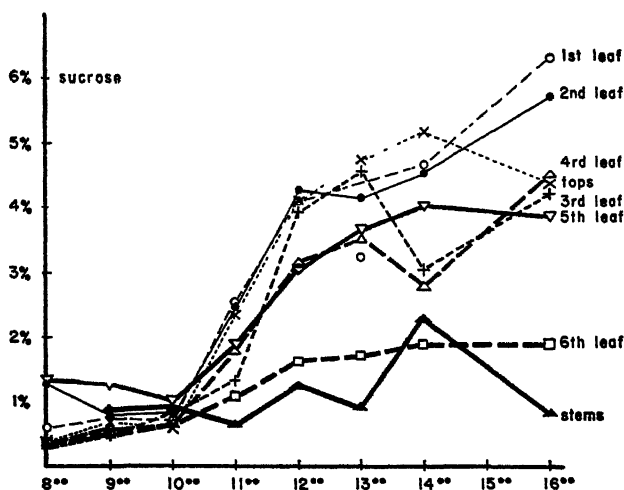


FIG. 1

Sucrose Content in Per Cent of Dry Weight of Whole Leaves and Stems of Tomato Plants, Placed at 8:00 in Natural Daylight at 18°C.

Samples collected each hour until 16:00 (4:00 P.M.)

× = sucrose content of top with youngest growing leaves; ○, ●, +, △, ▽, □, = sucrose content of youngest mature to oldest leaves respectively;

▲ = sucrose content of upper half of stem

Each sample mean of 7 plants

values for the 3rd and 4th leaves and upper stem sections. The leaves are much lower in sugar than expected, whereas the stems contain too much. This is probably not accidental, and the sucrose lost from the leaves seems to have turned up in the stems.

Whereas all the leaves show a consistent rise in sucrose content during the day, sucrose does not change in the stems (with the exception mentioned earlier). This was also true for the plants kept at

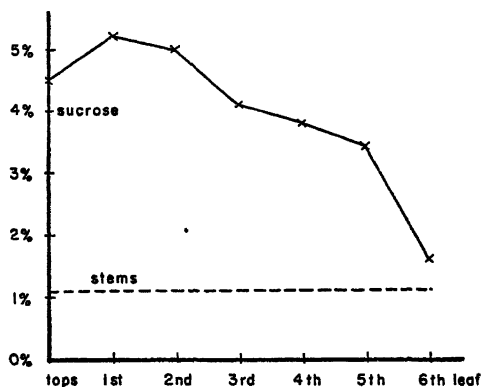


FIG 2

Sucrose Content in Afternoon of Tomato Leaves as a Function of Their Height of Insertion on Stem (1st Leaf is Youngest, 6th Leaf is Oldest)  
Same data as Fig. 1

26.5°C. in the light. Therefore little photosynthetic sucrose production occurs in stems.

The lag in sucrose formation is very marked during the first two hours of illumination. From the youngest to the oldest leaf a rapid rise in sucrose content occurs from 10:00 till noon and, after that, the rise is gradual and slight. This is clearly seen in Fig. 3, in which the

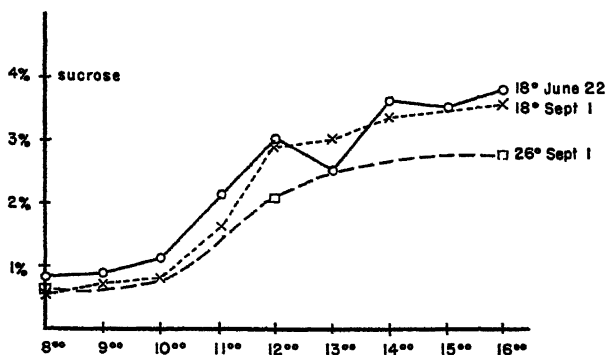


FIG. 3

Sucrose Content of Tomato Plants (Ordinate = Per Cent of Dry Weight) After Various Lengths of Exposure to Sunlight at Different Temperatures (○ and × at 18°C., □ at 26°C.) and on Different Days (○ on Cloudy Day, × and □ on Sunny Day)

mean sucrose concentration in all parts of the plant is plotted, doubling the weight of the stem values. In this graph also the mean sucrose content of the plants kept at 26.5°C. is shown; this remains consistently below that of the plants kept at 18°C. in the light, a phenomenon also noted in the first experiment. The latter is plotted as the mean sucrose content of the tops, 1st, 2nd, 3rd, 4th and 7th leaves, both in 26.5°

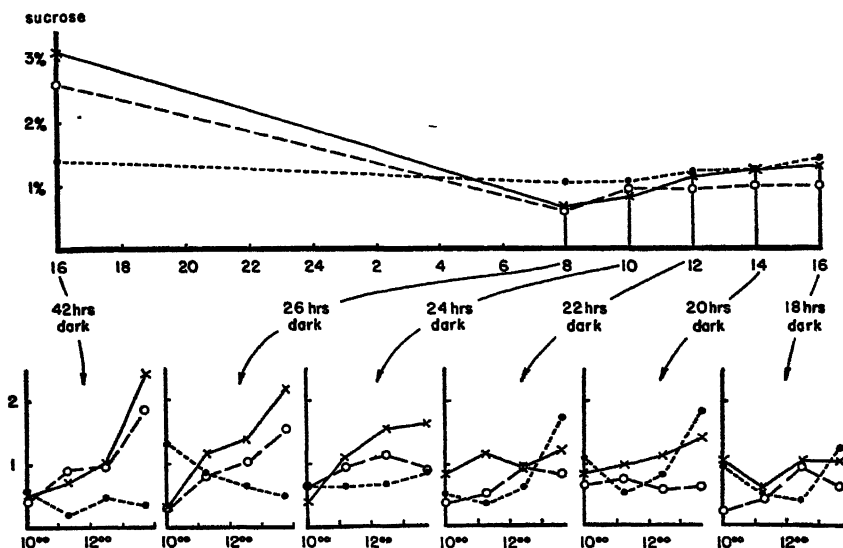


FIG. 4

Sucrose Content of Tomatoes, Kept from 8:00-16:00 Each Day in Full Daylight at 18°C., and from 16:00-8:00 in Darkness at 17°C. (upper curve)

At six times, marked by arrows, groups of 20 plants each were placed in darkness at 17°C., left there for the length of time indicated, and then all brought into the light at 10:00 at 18°C. The lower curves show their sucrose production in light.

- × — × sucrose content of 3 upper leaves
- — ○ sucrose content of 3 lower leaves
- - - - • sucrose content of stems

and 17°C. light temperature. Since the sucrose content of stems was not included, the whole curve lies slightly above that of the second experiment, but even though the light conditions were totally different (dark and cloudy in the first, full sun in the second experiment), the two curves are similar.

In another experiment the sucrose concentration in leaves and stems

was determined upon placing the plants in full sunlight after they had been kept for various periods in darkness. Fig. 4 gives most of the data. The upper curve is the sucrose content of control plants kept normally in the greenhouse at 18°C. during day and from 16:00 until 8:00 in the darkroom at 17°C. This curve is typical except for the slow rise

TABLE I

*C, H, N, Ash, Sucrose and Reducing Sugars in Per Cent of Dry Weight of Upper Leaves, Lower Leaves and Stems of Tomato Plants, Kept for 42 Hours in Darkness, and Then Exposed to Full Sunlight at 18°C.*

Material Collected at	Elementary Analysis			Micro Dumas Nitrogen	Carbon Content of Samples Reducing All Values to Constant		Sucrose Content per cent Dry Weight	Reducing Sugars
	Hydrogen	Carbon	Ash		Nitrogen Content	Ash Content		
Upper Leaves								
16:00 Oct. 24	5.84	38.61	15.57	4.83	38.6	38.6	3.16	3.44
10:00 Oct. 26	5.80	37.92	15.43	5.70	32.1	38.3	0.48	2.56
11:15 Oct. 26	5.54	37.59	17.92	5.33	34.0	32.7	0.68	3.28
12:30 Oct. 26	5.55	38.41	16.43	5.04	36.8	36.4	1.00	3.00
13:45 Oct. 26	5.52	37.46	16.20	5.14	35.2	36.0	2.42	3.88
Lower Leaves								
16:00 Oct. 24	5.45	34.64	21.19	4.14	34.6	34.6	2.58	3.44
10:00 Oct. 26	5.59	35.28	20.26	4.52	32.3	37.0	0.40	2.46
11:15 Oct. 26	5.43	35.13	22.04	4.48	32.5	33.8	0.86	3.10
12:30 Oct. 26	5.21	34.52	21.13	4.45	32.2	34.6	0.92	4.10
13:45 Oct. 26	5.00	34.23	22.23	4.49	31.5	32.6	1.86	3.48
Stems								
16:00 Oct. 24	4.87	31.92	22.68	4.06	31.9	31.9	1.40	3.78
10:00 Oct. 26	4.85	31.63	21.89	3.67	35.0	32.8	0.60	2.10
11:15 Oct. 26	4.63	31.18	24.15	4.12	30.7	20.3	0.20	2.70
12:30 Oct. 26	4.91	31.79	26.11	3.57	36.2	27.6	0.48	2.64
13:45 Oct. 26	4.62	30.37	21.10	3.84	32.1	32.6	0.36	1.80

in sucrose concentration in the leaves during an eight hour day. At the six points indicated (4:00 P.M., Oct. 24; 8:00, 10:00 A.M., Noon, 2:00 and 4:00 P.M., Oct. 25) groups of plants were taken out of the greenhouse and brought into the darkroom at 17°C. On October 26 all these plants were returned to full sunlight in the greenhouse kept at 18°C., and at intervals of 1½ hour, five plants were harvested and dried. The longer the plants had remained in darkness the lower was

the sucrose content of the upper leaves when they were taken out of the darkroom at 10:00 A.M. (of lower leaves and stems it was erratic). In the course of  $3\frac{1}{2}$  hours in light their sucrose concentration rose again as indicated by the lower graphs of Fig. 4.

The same dried material which was used as sample for the sucrose determinations, was analyzed for C, H, N and ash content. Dr. G. Oppenheimer carried out the elementary analyses and the Dumas nitrogen determination. Table I gives the results. The harvested tomato plants were not uniform enough to use the dry weight of the samples as a basis for calculation of increase in carbon due to assimilation in light. Therefore, the nitrogen values were used as reference

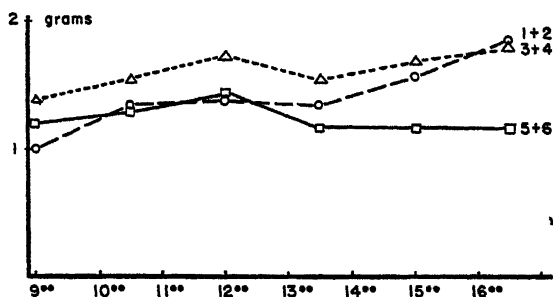


FIG. 5

Dry Weight of Leaves Harvested at  $1\frac{1}{2}$  Hour Intervals After They Have Been Placed in Full Daylight at 9:00 at 18°C. (same plants as analyzed in Fig. 6)

- — ○ Weight of two youngest leaves
- △ — — — — △ Weight of two medium leaves
- — — — — □ Weight of two oldest leaves

values, assuming that these changed least during the light and dark periods. During darkness the nitrogen values increase relative to the carbon and hydrogen values. In light the reverse process occurs. By calculation we find that the change in C content during 42 hours in darkness equals 6.5 and 2.3% for upper and lower leaves. The change in sugar content (sucrose and reducing sugars) accounts for only 1.5 and 1.3%. The discrepancy between the two methods of determination of the metabolism can mean:

- a. Nitrogen as the reference value for the carbon content is inadequate. The ash content, however, seems much worse as reference value.
- b. Products other than sugars are formed and metabolized.



Although the latter possibility is not in keeping with Smith's (1944) results (it was found previously (Went 1944) that no starch was formed by tomatoes), this seems to bear out the rather consistent increase in dry weight of leaves harvested later in the day. Fig. 5 shows how the youngest leaves increased most in dry weight from 9:00 until 16:30,

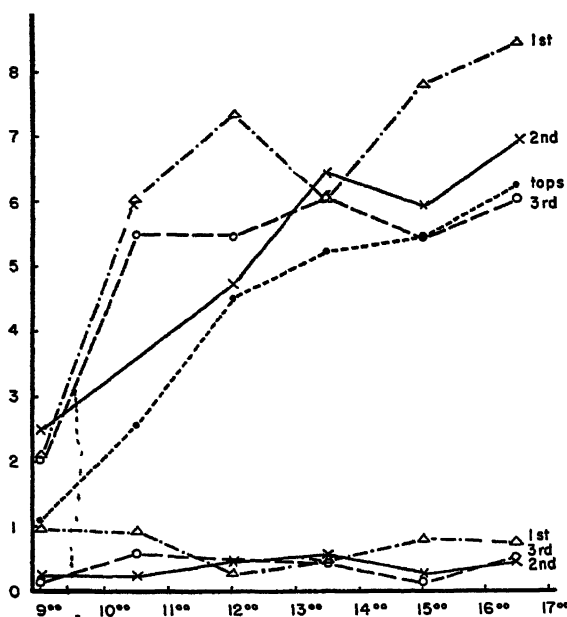


FIG. 6

Sucrose Content (in Per Cent of Dry Weight) of Leaf Blades and Petioles Harvested Separately

Upper four curves: sucrose in top and in leaf blades.

Lower three curves: sucrose in petioles

△- - - -△ sucrose in blades and petioles of youngest full grown leaves

×- - - -× sucrose in blades and petioles of second youngest leaves

○- - - -○ sucrose in blades and petioles of third youngest leaves

while the oldest leaves did not show any appreciable over-all increase. The increase in weight of the upper and middle leaves was much greater than the increase in sugars could account for. Therefore, it seems likely that, in addition to sugars, other substances are metabolized in tomato leaves.

Some experiments were carried out to study the fate of the sucrose in other parts of the tomato plant during both day and night. Fig. 6 shows the sucrose content of leaf blade and petiole when these are harvested separately. The leaf blades show the usual rapid increase in the forenoon, whereas the petioles do not change at all. Therefore all photosynthetic sucrose production must have occurred in the leaf

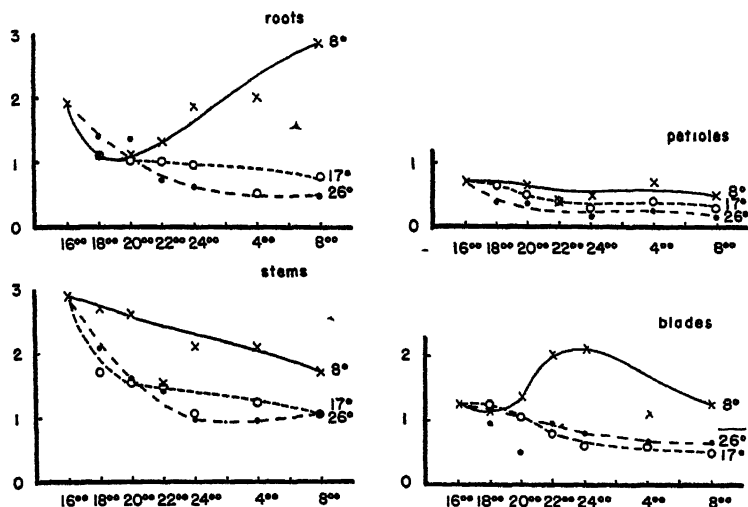


FIG. 7

Sucrose Content (in Per Cent of Dry Weight) of Tomato Leaf Blades (Lowest Curve), Petioles, Stems and Roots (Upper Curve) When Harvested at Several Intervals During Their Stay in Darkness at 8°, 17° and 26°C.

At 16:00 they were removed from greenhouse at 18°C. Mean values for the upper three younger leaves, whole stems, and whole roots

- X—X plants kept at 8°C.
- O---O plants kept at 17°C.
- - -● plants kept at 26°C.

blade, whose sucrose content rose to 8%, which is higher than the values shown in Figs. 1 and 3, since the low sucrose content of the petioles did not decrease the sucrose content of the leaves.

In one experiment well over 100 tomato plants, all kept at 18°C. in full sunlight, were divided into three groups, which were placed at 16:00 in 26°, 17° and 8°C. darkness. At regular intervals samples of 5

plants each were harvested, to follow the sucrose movement during night. The results are shown in Fig. 7. The lowest graph (7a) represents the sucrose content of the leaf blades of the 3 youngest full grown leaves. In the latter part of the night the leaves kept at 26°C. had consistently more sucrose than those kept at 17°C., which confirms the earlier results (Went 1944). In the 8°C. night, first a rapid rise in sucrose is followed by a strong decrease. Obviously temperature has a dual effect; first, liberation of sucrose from some stored form, and later, removal of this sucrose. This dual effect makes interpretation of the data very uncertain. Yet a fair estimate of the transport of sucrose out of the tomato leaf can be made by contrasting the sucrose values for the first half of the night with those for the second half. This is done in Table II. The difference can be ascribed to transport of

TABLE II

*Sucrose Content in Per Cent of Dry Weight of the Leaf Blades of the  
3 Youngest Full Grown Leaves*

(Means of the plants collected at 18:00, 20:00, 22:00 and 24:00 are contrasted against the mean of the collections at 4:00 and 8:00 later that night.)

Night Temperature	26°	17°	8°
Sucrose content 18:00-24:00	0.79	0.90	1.63
Sucrose content 4:00- 8:00	0.67	0.52	1.14
Difference (translocation)	0.12	0.38	0.49

sucrose out of the leaf blade, and this is greatest at the lowest temperature, as originally found with other methods (Went 1944).

The next higher set of curves (7b) show that the changes in sucrose content of the petioles are not great, just as found in Fig. 6.

In the stems (Fig. 7c) a great reduction in sucrose content takes place in the early night; this is least at the lowest temperature.

In the roots (Fig. 7d) the situation is just the opposite from that in the leaves. At the lowest temperature there is first a rapid decrease in sugar content, but later the sucrose increases. At the highest temperature the sucrose content drops most. Since the increase in sucrose content of the roots parallels the rate of sucrose loss in the leaves (Table II), it is another indication that the sucrose found in the roots is derived from the leaves, and that the rate of sugar translocation in a tomato plant decreases as the temperature rises from 8° to 26°C. (Went, 1944).

## DISCUSSION

Photosynthetic sucrose formation does not necessarily follow photosynthetic  $\text{CO}_2$  reduction. Although, in the tomato, sucrose is the only carbohydrate which shows daily fluctuations in concentration (Went, 1944), and although in the sunflower 98% of all  $\text{CO}_2$  reduced in light can be accounted for as carbohydrate (Smith, 1944), the data of Table I and Fig. 5 suggest that compounds other than sugar are formed in light. This can be calculated in yet another way. In a number of fast-growing plants the rate of  $\text{CO}_2$  reduction in light per unit leaf area strives towards a maximal rate, which is about 0.25 mg.  $\text{CO}_2$  reduced per hour per square centimeter (in sugar cane values of

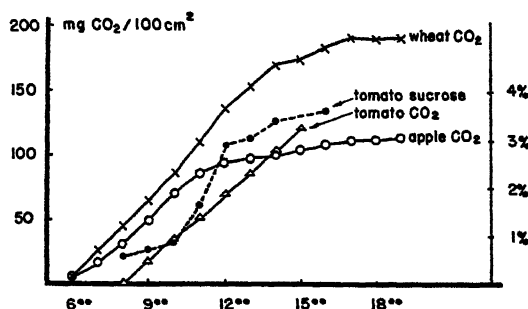


FIG. 8

Comparison Between Total  $\text{CO}_2$  Reduction (Left Ordinate, in mg. per 100 cm.<sup>2</sup> Leaf Surface) and Sucrose Production (Right Ordinate in Per Cent of Dry

Weight) as Found in Tomato Leaves = ●-----●

Data of Kostytchew et al. (1930) for wheat = X———X; of Kurssanow (1933) for apple = O-----O, and Mitchell (1937) for tomato = Δ-----Δ

0.5 mg. were found, McLean, 1920). The maximal amount reduced by wheat over a whole day was 2.0 mg. (Kostytchew and Kardo-Syssoiewa, 1930; see Fig. 8). In tomatoes Mitchell found 1.2 mg. in 7 hours illumination with an intensity near saturation. For apple leaves this value was 1.1 mg. in a whole day (Kurssanow, 1933; Fig. 8). If this were all converted to carbohydrate an increase in weight of 0.7–1.3 mg. dry weight per cm.<sup>2</sup> leaf area could be expected, or, since in tomatoes the dry weight per cm.<sup>2</sup> equals about 2.3 mg., an increase in dry weight of 35–55%. From Fig. 5 an increase of 35–85% can be calculated. Since the increase in sucrose is only 8% at most, sucrose

production can account for only 10–20% of the total increase in dry weight.

If it is conceded that sucrose represents only a minor part of the products formed by photosynthesis, there must be an equilibrium between sucrose and the other photosynthetic products, otherwise the curve for sucrose formation would not follow so closely the rate of photosynthesis (see Fig. 8). In the early afternoon photosynthetic  $\text{CO}_2$  reduction decreases and practically stops, sucrose formation comes to a halt, and effective photosynthesis as judged by total plant weight stops at about the same time (Went, 1945).

Is the 8% sucrose, based on dry leaf blade weight, not very low as the maximal sugar concentration, which can be attained in a leaf? Since the dry weight is about 13% of the wet weight, the actual overall sucrose concentration in the leaf blade reaches only 1.0%. But part of the blade, such as veins and epidermis, presumably does not contribute to the sucrose production so that we can roughly estimate the sucrose concentration in the palisade and spongy parenchyma to reach 2%. This sucrose is formed in or near the chloroplasts; at least inside the cytoplasm. All osmotic investigations have shown a very low permeability of the tonoplast for sucrose. Since the sucrose content of tomato leaves increases so rapidly during day and decreases equally rapidly during night, it is unlikely that much of the photosynthetic sucrose ever reaches the vacuole.

The cytoplasm in palisade and spongy parenchyma does not occupy more than 10 to 15% of the cell volume, so that we come to the conclusion that the actual sucrose concentration in the cytoplasm must reach somewhere between 14 and 20% on a wet weight basis. It is quite possible that 20% sucrose is the highest concentration at which the photosynthetic process can proceed; beyond that chemical processes might either be inhibited or slowed down by the accumulation of reaction products (for experimental evidence see Kursanow, 1933). These possibilities satisfactorily explain the curve of photosynthetic sucrose formation (Figs. 1, 3, 6 and 8), which all reach a final level asymptotically.

It should be pointed out that this 14–20% sucrose, as calculated to be produced in the cytoplasm, corresponds with the 15–20% sucrose concentration as found in the sieve tubes, so that it is quite possible that the sieve tube contents are pure cytoplasmic sap, and that sucrose is not concentrated as it enters the sieve tubes. This makes it possible

to conceive the movement of sucrose from leaf cells to sieve tube as a diffusion or streaming process along a concentration gradient. It also would remove the objections raised by so many investigators against the theories of sugar transport of Mason and of Münch, namely, that the soluble sugar concentration in receiving organs so often is higher than in the supplying tissues. But in both sugar cane and sugar beets the sucrose content of the storage tissue never exceeds 20%, which is another reason to assume that 20% sucrose is a critical value, reached in cytoplasm, in sieve tubes and in storage tissue. By assuming that this is the maximal concentration to which photosynthesis can build up sucrose, and that this concentration is passed on from cell to sieve tube to storage tissue without becoming diluted or side-tracked into vacuoles on its way, a great simplification is achieved in the auxiliary hypotheses necessary to explain sugar transport inside the plant. At the same time this simple assumption, based on experimental evidence, removes some of the most serious objections against current theories of sugar transport.

### SUMMARY

The sucrose content of leaves, petioles, stems and roots of tomato plants has been followed in the course of 24 hours. During the day the sucrose content of petioles and stems remains fairly constant, but in leaf blades it increases rapidly during the forenoon, slowly during the afternoon. During the night the sucrose disappears again from the leaves, and can be retrieved partially in the roots. The significance of these data in relation to theories of sugar translocation is discussed.

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# The Fatty Acids of Human Milk Fat \*

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## INTRODUCTION

Two years ago, when the present investigation was begun, no accurate quantitative data were available on the component fatty acids of human milk fat. The most detailed investigation of the nature of the acids of this fat had been reported from this Laboratory by Bosworth (1) in 1934. Earlier papers concerned with the composition of this fat were by Ruppel (2), Laves (3), Arnold (4), Bardisian (5), Polonovski (6) and Roller (7). Fatty acids recognized before Bosworth's work were the usual saturated acids from butyric to stearic and oleic acid. Bosworth presented evidence for the following additional acids: decenoic, tetradecenoic, hexadecenoic, linoleic and arachidonic. He also inferred the presence of several acids of carbon series above  $C_{18}$ . Linoleic and arachidonic acids were identified by isolation of their characteristic bromine addition products.

In 1944 two papers appeared describing quantitative analyses of the component acids of human milk fat. The first of these, by Hilditch and Meara (8), reported analyses of two specimens in early lactation and two in late lactation. The second paper, by Baldwin and Longenecker (9), included results on two specimens taken during the first three days of lactation and on one mature specimen. A later paper from Hilditch's laboratory (10) includes an analysis of an additional specimen in connection with a detailed evaluation of glyceride structure. In Table I we have summarized these results, and have included for purposes of comparison our analysis, described later in the present report.

The results reported from the Hilditch and the Longenecker laboratories differ chiefly in the following respects. The former group found no acids below  $C_{10}$  and

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\*\* S.M.A. Research Assistant in Physiological Chemistry.



TABLE I  
*The Component Fatty Acids of Human Milk Fat*

As Esters	Baldwin and Longenecker (9)			Hilditch and Mearns (8, 10)					Present Work	
	1st and 2nd days	3rd day	Ma- ture	Early (F.A.)	Early (Fat)	Full (F.A.)	Late (Fat)	(Fat)	I	II
Iodine No.	—	—	—	56.0	52.1	54.7	48.2	60.1	61.7	
	<i>All values in weight per cent</i>									
Butyric	0.2	0.3	0.4	—	—	—	—	—	tr. <sup>2</sup>	tr. <sup>2</sup>
Caproic	0.1	0.1	0.1	—	—	—	—	—	—	—
Caprylic	0.5	0.1	0.3	—	—	—	—	—	tr.	0.1
Capric	3.5	0.9	2.2	2.7	0.8	1.7	0.5	1.4	1.4	2.1
Lauric	0.9	2.6	5.5	5.1	6.1	6.4	7.0	5.9	5.4	4.8
Myristic	2.8	4.9	8.5	8.1	10.8	7.6	13.9	7.9	6.7	6.0
Palmitic	24.0	27.8	23.2	22.5	24.6	22.4	24.1	23.0	21.0	21.6
Stearic	9.9	7.7	6.9	8.3	7.3	9.0	9.6	7.0	6.8	6.8
Arachidic	4.9	2.7	1.1	1.0	1.8	0.9	—	1.1	See (1)	See (2)
Decenoic	0.2	0.1	0.1	tr.	tr.	tr.	tr.	—	tr.	tr.
Dodecenoic	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	tr.
Tetradecenoic	0.1	0.2	0.6	1.3	0.4	0.5	0.9	0.7	0.7	0.4
Hexadecenoic	1.8	2.9	3.0	3.1	3.3	3.7	2.8	3.3	2.7	2.6
Oleic (Octadecenoic)	36.0	37.1	36.5	36.4	32.8	36.6	30.2	35.6	39.1	39.5
Octadecadienoic	7.5	6.2	7.8	7.9	6.3	8.2	5.5	7.8	10.3	10.4
Octadecatrienoic	0.3	0.3	0.4	—	—	—	—	—	0.5	0.5
Octadecatetrenoic	—	—	—	—	—	—	—	—	0.2	0.2
Arachidonic	1.8	1.6	0.9	—	—	—	—	—	0.8	0.8
C <sub>20</sub> and Above Unsat'd	4.6 <sup>(1)</sup>	4.7 <sup>(1)</sup>	2.4 <sup>(1)</sup>	3.5	5.7	2.9	5.4	4.1	4.3 <sup>(1)</sup>	3.6 <sup>(2)</sup>

<sup>(1)</sup> Both saturated and unsaturated. Includes 1.6% C<sub>20</sub>, 1.7% C<sub>22</sub>, and 1.0% C<sub>24</sub>.

<sup>(2)</sup> Both saturated and unsaturated. Includes 1.1% C<sub>20</sub>, 1.9% C<sub>22</sub>, and 0.6% C<sub>24</sub>.

<sup>(3)</sup> Reported as eicosadienoic.

reported the presence of linoleic acid. Baldwin and Longenecker, on the other hand, found 0.8% of acids below C<sub>10</sub>, including 0.4% of butyric acid, but, with the small quantities of material at their disposal, they could identify no linoleic acid by isolation of the characteristic tetrabromide, although octadecadienoic acid was demonstrated spectroscopically, as well as octadecatrienoic acid and probably higher acids of these types.

In the present work we planned to secure a large specimen of milk fat, so that in the course of application of the ester-distillation method we would have sufficiently large main fractions available to permit their resolution by the low temperature crystallization procedures described in a previous analysis of the acids of human body fat (11). Thus, our two analyses are based on distillations of 398 and 337 g. batches of esters, respectively, of the same fat specimen. For several reasons we believe our second analysis to be somewhat more accurate.

In addition to applying the ester fractionation procedure to two

specimens of the fat and the crystallization method to the main fractions we have employed the spectroscopic method, after alkali isomerization, to the original fat, to the mixed esters and to the  $C_{18}$  fraction. We were unable to find more than traces of acids below  $C_{10}$ , as noted by Hilditch and Meara. With reference to linoleic acid, less than half of the octadecadienoic acid was shown to be linoleic acid, but this latter acid was definitely identified, thus confirming Bosworth's results. The presence of trienoic acids, both in the  $C_{18}$  and in the higher series, was verified spectroscopically but, with respect to the former, no appreciable amounts of linolenic acid were found. Evidence was presented for the presence of an octadecatetrenoic acid. All of the analyses in Table I confirm the presence of arachidonic acid as first reported by Bosworth.

## EXPERIMENTAL

### *Description of Specimen*

We are greatly indebted to Dr. David W. Goltman and to Mrs. Louise Schwartz of the Variety Club Mother's Milk Bank, The John Gaston Hospital, Memphis, Tennessee, for their interest and cooperation in the present work; and to Mrs. Schwartz, especially, for her aid in connection with the collection and shipment of 52 liters of human milk. This milk was collected from colored and white mothers,—about 3/4 from colored women, at various stages of lactation. It was frozen at the time of collection, and was shipped to us in that state in a special container, packed in dry ice. It was shipped in three lots, collected over three periods. Upon arrival in our laboratory the milk was melted and passed twice through a hand centrifugal separator. Attempts to separate the butter fat from the cream by churning failed, but after shaking for four hours at 15–20°C., a fat layer formed readily when warmed on the steam bath. This layer was separated, water was removed by warming under reduced pressure, and curd was removed by filtering while warm. The yield of fat from the first 10 gals. of milk was 1141 g. The total final specimen available for investigation amounted to 1300 g.

Analytical data on the final composite specimen were as follows: Iodine No., 61.65; Sap. No., 204.7; Reichert-Meissl No., 0.64;  $n_D^{25}$ , 1.4598; m.p., 26–26.5°; unsaponifiable, 0.46%. The iodine number of this specimen is even higher than that reported by Hilditch and Meara, 60.1, in their latest work (10) and is considerably higher than the values usually reported for human milk fat, 50–55. It was to be expected, therefore, that somewhat larger quantities of the principal unsaturated acids would be found in this specimen than in specimens of lower iodine number.

### *Preparation and Distillation of Methyl Esters*

In applying the ester fractionation procedure, Baldwin and Longenecker first removed phospholipids from the fat, removed steam-

volatile acids, and prepared and distilled the methyl esters of the non-volatile acids. Their results were obtained on relatively small specimens of fat (20–46 g.), and are a reflection of the remarkable efficiency of manipulation achieved in their laboratory. Hilditch and Meara worked with considerably larger specimens of milk fat and fatty acids. After steam distillation, they separated the non-volatile acids into saturated and unsaturated acids, prepared the methyl esters of each group separately, and distilled them, which is the usual procedure employed by this group.

Our procedure differs from either of the above, in that we prepared the methyl esters directly from the whole fat by the usual method and made every endeavor to recover low boiling material, as noted later. We did not separate the acids into saturated and unsaturated, but, rather, prepared main fractions, as far as possible, and calculated their composition from molecular weights and iodine numbers, and, in the  $C_{18}$  fraction, by other procedures. Further, our main fractions were examined by low temperature crystallization.

In our first ester-distillation run, 423.5 g. of the fat was refluxed 44 hrs. with 1200 cc. of acidified C.P. methanol. About 800 cc. of the methanol was distilled off at ordinary pressure, and 1500 cc. of water was added to the residual esters.

After shaking, the ester layer was separated and transferred to a 1000 cc. Claisen flask. Remaining water and alcohol were removed by warming under reduced pressure, alcohol and low-boiling distillate being caught in a trap cooled with dry ice. The residual crude esters, 421.4 g., were then distilled into a flask cooled with ice water, again protected against loss by the use of a trap cooled with dry ice. The residue from this distillation amounted to only 2.4 g., and was assumed to be composed mainly of unsaponifiable. The distilled esters amounted to 417.4 g.; their constants were: Iodine No., 61.7; m.w., 273.8; SCN No., 50.8; polybromide No., 0.79. By the use of a curve showing the relationship between the weight of methyl arachidonate and the yield of methyl octabromarachidate upon bromination, this material was shown by interpolation to contain 1.0% of methyl arachidonate.\*

The alcohol distillates, water layer removed above, and contents of the traps, and also 2.0 g. of material, recovered later in a trap during the distillation of these esters, were combined, an excess of KOH added, and, after saponification by boiling for 12 hours, the alcohol and water were removed by heating under reduced pressure. The residue was acidified with 50% sulfuric acid, and exhaustively steam distilled. From the aqueous residue 2.4 g. of insoluble fatty acids were recovered of molecular weight 265.9. The steam distillate was repeatedly extracted with ether. The residual

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\* Data for this curve, and also for one showing the relation between yields of hexabromide from varying amounts of linolenic acid, were obtained by Miss Mary Frechtling, and are to be published soon.

water solution was titrated with 0.1 *N* NaOH, the result being equivalent to 8 mg. of butyric acid, although the odor of this acid could not be detected. The ether extract was dried over sodium sulfate, the ether distilled off and the residual acids distilled from a small Claisen flask fitted with an indented side arm. The distillate amounted to 0.5 g. of m.w. 189.3, and the residue 1.0 g. of m.w. 198.2. The ether from this distillation, as well as the sodium sulfate used in the drying, was shaken with water and titrated with alkali. Titration values corresponding to 40 mg. of butyric acid were obtained, although again there was no evidence of the odor of this acid. Assuming the total recovery of butyric acid to be 48 mg., as indicated above, this amount is still only about 0.01% of the original esters. The several fractions of higher acids, recovered in the course of these manipulations, were apportioned on the basis of molecular weight to the several carbon series in the course of calculating from the results of the main distillation which is now to be reported.

#### *Distillation of the Methyl Esters of Human Milk Fat Fatty Acids and Analyses of the Fractions*

A charge of 398.2 g. of the esters was fractionally distilled through the efficient column in use in this laboratory. As a result of this distillation there were available thirteen fractions and residue ( $R_1$ ), the latter including the column hold-up. After analysis of  $R_1$ , the remainder of this fraction was redistilled from a small Claisen flask with a special indented side arm. The weights of the resulting six fractions and residue  $R_2$  were corrected to account for the original 43.0 g. of  $R_1$ . Results of the two distillations with fraction analyses are shown in Table II.

In Table II fraction 4 was considered to be pure  $C_{12}$  material, fraction 8 as  $C_{16}$  and 10, 11, 12 and 13 as  $C_{18}$  esters. Other fractions were apportioned to the several carbon series on the basis of molecular weight. Thus, for example, the 43.0 g. of  $R_1$ , from analysis of the fractions and  $R_2$ , was found to be composed of  $C_{18}$ , 23.0 g.;  $C_{20}$ , 9.4;  $C_{22}$ , 6.7 and  $C_{24}$ , 3.9 g. As the iodine numbers of the several  $R_1$  fractions were approximately those of monoethenoic esters, calculations were based on the theoretical molecular weights of the monoethenoic esters of these series.

The second ester preparation from 345 g. of fat and distillation of the resulting esters were carried out as described above, except that the esters recovered in the dry ice trap in the course of the main distillation were analyzed directly, instead of being added to the low molecular weight acids. A somewhat larger recovery of "butyric acid" was achieved in this operation,—200 mg. This amount is still only 0.06% and its identity as butyric acid is considered questionable.

Results of calculations of the amounts of the several carbon series

TABLE II

*Results of Fractionation of the Methyl Esters of Human Milk Fat and Fraction Analyses*

Fraction	°C. Main Distillation	Wt. g. 398.2 g.	Iodine No.	Mol. Wt. Esters	C Series
1	66-74	2.9	1.9	184.3	C <sub>5</sub> -C <sub>10</sub>
2	74-94	2.8	1.9	198.9	C <sub>10</sub> -C <sub>12</sub>
3	94-97	8.4	1.3	210.0	C <sub>10</sub> -C <sub>12</sub>
4	97	4.7	2.1	213.2	C <sub>12</sub>
5	97-120	9.4	3.2	220.2	C <sub>12</sub> -C <sub>14</sub>
6	120-3	31.0	10.0	246.5	C <sub>14</sub> -C <sub>16</sub>
7	123-41	8.2	20.0	265.4	C <sub>14</sub> -C <sub>16</sub>
8	141-4	76.6	10.9	269.4	C <sub>16</sub>
9	144-55	12.1	76.2	286.7	C <sub>16</sub> -C <sub>18</sub>
10-11	155-7	167.2	95.3	295.0	C <sub>18</sub>
12	157-63	19.8	69.4	295.9	C <sub>18</sub>
13	163	8.0	77.9	296.4	C <sub>18</sub>
R <sub>1</sub>		43.0	92.7	312.7	C <sub>18</sub> -C <sub>24</sub>
Total		394.1 <sup>*</sup>			
Redistillation of R <sub>1</sub>					
R <sub>1</sub> -1		4.5	76.2	299.9	C <sub>18</sub> -C <sub>20</sub>
R <sub>1</sub> -2		13.8	78.1	300.9	C <sub>18</sub> -C <sub>20</sub>
R <sub>1</sub> -3		10.6	89.7	304.7	C <sub>18</sub> -C <sub>20</sub>
R <sub>1</sub> -4		3.5	117.2	321.1	C <sub>18</sub> -C <sub>20</sub>
R <sub>1</sub> -5		3.0	128.6	360.6	C <sub>22</sub> -C <sub>24</sub>
R <sub>1</sub> -6		1.6	113.9	360.6	C <sub>22</sub> -C <sub>24</sub>
R <sub>2</sub>		6.0	41.2	363.8	C <sub>22</sub> -C <sub>24</sub>
Total		43.0			

<sup>\*</sup> An additional 4.1 g. was recovered in the course of working up the original esters, as explained in the text. The amounts of acids thus recovered were assigned to the appropriate carbon series on the basis of the molecular weights.

of this specimen of human milk fat from the analyses of the fractions obtained from the two distillations are shown in Table III.

#### *Resolution of Ester Fractions by Low Temperature Crystallization*

Percentages of decenoic, dodecenoic, tetradecenoic and hexadecenoic acids (esters) in Table I were calculated from the iodine numbers of the several fractions, on the assumption that monoethenoic esters are the only unsaturated esters present in these series. In order to verify the presence of these unsaturated acids and to study the C<sub>18</sub>

TABLE III  
The Carbon Series of Human Milk Fat

Series	Distillation	
	I	II
	Weight Per Cent	
C <sub>8</sub>	tr.	0.1*
C <sub>10</sub>	1.4	2.1
C <sub>12</sub>	5.5	4.8
C <sub>14</sub>	7.4	7.0
C <sub>16</sub>	23.7	24.2
C <sub>18</sub>	56.9	57.4
C <sub>20</sub>	2.4	1.9
C <sub>22</sub>	1.7	1.9
C <sub>24</sub>	1.0	0.6

\* 0.03 and 0.06% respectively.

series and above more fully, the main fractions, *i.e.*, those which represent ester mixtures of almost entirely one carbon series, have been separated into their component esters by low temperature crystallization.

*The C<sub>12</sub> Esters.* Fractions 3 and 4 of Table II and a corresponding fraction from distillation II were combined and treated by the procedure described in Chart 1.

Chart 1	
Crystallization of C <sub>12</sub> Esters	
C <sub>12</sub> esters	
11.9 g. in 900 cc. MeOH	
Cooled to - 70°C.	
↓	
C <sub>1</sub> : 11.6 g.	F <sub>1</sub> : 0.3 g.;
Saponified.	Iodine No., 28.8;
11.4 g. acids.	m.w., 214.5.
Twice crystallized from	Composition:
550 cc. petrol ether at - 35°C.	24% dodecenoate;
↓	
C <sub>2</sub>	F <sub>2</sub> : 1.7 g.;
9.4 g. lauric acid.	Iodine No., 7.3;
Iodine No., 0.7; m.w., 199.1;	m.w., 207.1.
m.p., 43.3-43.5°	

The presence of dodecenoate in the C<sub>12</sub> fraction is fully substantiated by the data in Chart 1. The molecular weights of F<sub>1</sub> and F<sub>2</sub> indicate

the presence of small amounts of  $C_{14}$  acids in the fractions studied.  $C_2$  is lauric acid of about 99% purity.

*The  $C_{14}$  Esters.* The  $C_{14}$  main fractions of the two distillations were combined and crystallized as described in Chart 2. It is to be noted that fraction 6 contains about 14% of  $C_{16}$  esters.

Chart 2  
*Crystallization of  $C_{14}$  Fractions*

Fraction 6 (1st dist.): Iodine No., 10.0; m.w., 246.5

Fraction 5 (2nd dist.): Iodine No., 6.6; m.w., 241.9

38.8 g. in 2500 cc. MeOH.

Cooled to  $-60^{\circ}\text{C}$ .



$C_1$   
Added 2500 cc. MeOH  
Cooled to  $-60^{\circ}\text{C}$ .

$F_1$ : 3.5 g.;  
Iodine No., 73.2.  
2.4 g. tetradecenoate;  
1.1 g. myristate.



$C_2$ : 33.4 g.;  
Iodine No., 0.1;  
m.w., 246.6.  
86% myristate;  
14% palmitate.

$F_2$ : 1.4 g.;  
Iodine No., 58.2.  
0.8 g. tetradecenoate;  
0.6 g. myristate.

Calculated from the iodine numbers of  $F_1$  and  $F_2$  in Chart 2, 3.2 g of methyl tetradecenoate was recovered, or approximately 8.3% of the  $C_{14}$  esters. The total amounts of  $C_{14}$  esters found in the two distillations were 7.4 and 7.0% respectively; applying the percentage of 8.3 to the two values gives 0.61 and 0.58% tetradecenoate for the two runs, whereas by direct calculations from the iodine numbers the values were 0.7 and 0.4% respectively. These latter values are considered to be more accurate and are recorded in Table I.

*The  $C_{16}$  Esters.* The  $C_{16}$  main fractions of the two distillations were crystallized separately with almost identical results. Chart 3 describes the procedure which was used for the resolution of the esters of the  $C_{16}$  fraction of the second distillation.

The results in Chart 3 show a remarkably efficient separation of the methyl esters of palmitic and hexadecenoic acids. Thus, in two crystallizations most of the material has been separated into and isolated as nearly pure methyl palmitate and 95.2% methyl hexadecenoate. The hexadecenoate, calculated from the iodine numbers, amounts to 10.8% of the material crystallized. This percentage multiplied by the total

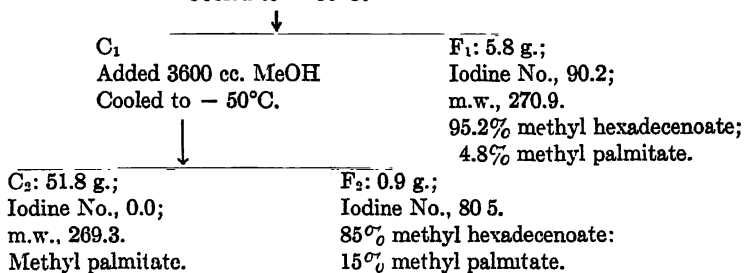
## Chart 3

*Crystallization of C<sub>16</sub> Esters*

59.1 g.; Iodine No., 10.2; m w., 268.8.

Dissolved in 3600 cc. MeOH.

Cooled to - 50°C.



C<sub>16</sub> fraction of Table III (24.2%) gives 2.6% of the original esters which agrees exactly with the value reported for this ester in Table I, which was obtained by calculation of the hexadecenoate from the iodine numbers of the original fractions.

The methyl palmitate of Chart 3 was combined with the corresponding fraction resulting from crystallization of the C<sub>16</sub> fraction from the first distillation. Part of this material was redistilled yielding pure palmitate of Iodine No. 0.0; m.w. 270.0; and m.p. 29.8°C. The remainder, 40.0 g., was saponified, and the acid was crystallized once from 1500 cc. acetone at - 20°C. The resulting crystals of pure palmitic acid analyzed as follows: Iodine No. 0.0; m.w. 255.6; m.p. 63.0–63.4°. The hexadecenoate fractions from the two crystallizations were combined, saponified and the resulting hexadecenoic acid distilled, yielding 8.8 g. of acid: Iodine No. 94.8; m.w. 255.5; m.p. - 1.6 to 1.4°. This melting point agrees with the value found for 9,10-hexadecenoic acid, isolated in a somewhat purer state from menhaden oil by Smith and Brown (12).

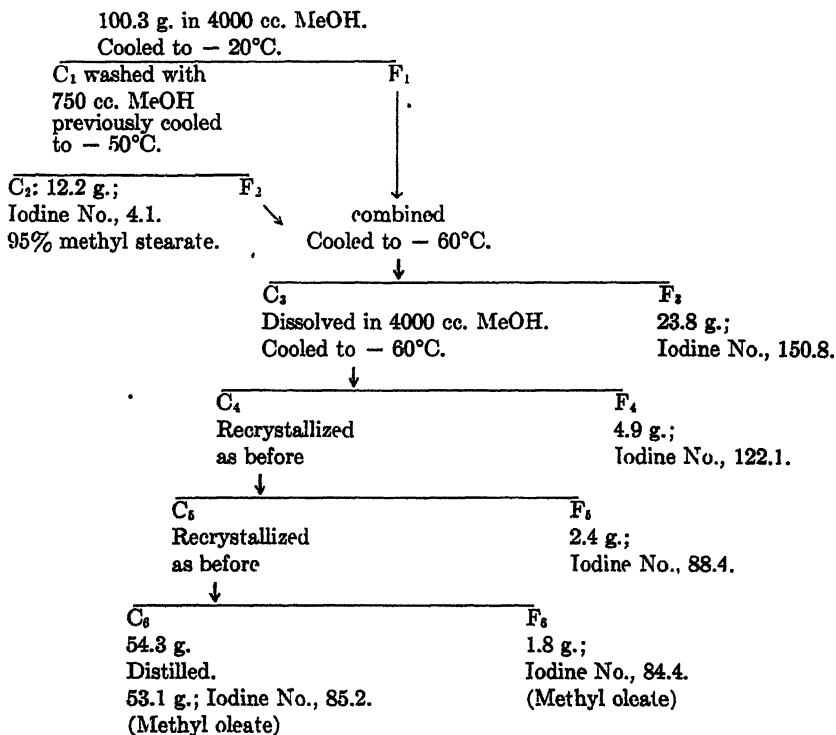
*The C<sub>18</sub> Esters.* As we wished to study the unsaturated acids of this series in great detail, the several fractions from the two distillations, containing only C<sub>18</sub> esters on the basis of molecular weight, were combined. Our results with respect to C<sub>18</sub> acids in Table I are based on data obtained on this composite sample, rather than on the respective fractions from each run. Differences in Table I with respect to this series are due therefore to differences in recovery in the two distillations, as noted in Table III, namely 56.9 and 57.4% respec-



tively. As a result of the decision to follow this course we had available about 325 g. of the esters in question. In distillation I the fractions used (Table II) were 10, 11 and 12. The composite specimen gave the following constants: Iodine No., 93.4; SCN No., 77.8; ether-insol. bromides, 0.0.

A specimen of the  $C_{18}$  esters was partially separated into its components by the procedure described in Chart 4.

Chart 4  
*Crystallization of  $C_{18}$  Esters*



The composition of  $C_2$  in Chart 4 was calculated as a mixture of stearate and oleate; of  $C_6$  and  $F_6$  as oleate and of  $F_3$ - $F_6$  inclusive as binary mixtures of oleate and linoleate. The total recovery was 99.4 g. Such a calculation shows the  $C_{18}$  esters of this specimen of fat to consist of: stearate, 11.8; oleate, 68.1; and linoleate, 20.1%. A second

batch of esters, crystallized by a similar, but not identical procedure, gave the following composition: stearate, 11.4; oleate, 68.6; and linoleate, 20.0%. The average result by this method was: stearate, 11.6, oleate, 68.3; and linoleate, 20.1%. The composition of this material was further evaluated from the thiocyanometric equations of Cramer and Brown (11) with these results: stearate, 10.2; oleate, 70.5; and linoleate, 19.3%. In the above calculations from both crystallization and thiocyanometric data, the assumption was made that no trienoic or tetrenoic esters were present, the assumption being based on obtaining no ether-insoluble bromides when the  $C_{18}$  esters were brominated.\*

In view of the fact that Baldwin and Longenecker found a small amount of octadecatrienoic acid in human milk fat spectroscopically, the importance of testing the present specimen of  $C_{18}$  esters by this method was obvious. Since we are not at present equipped for this work, we were glad to accept the offer of Drs. Kraybill and Beadle, of the American Meat Institute Laboratory, Chicago, Illinois, to carry out this analysis, as well as the examination of the original fat and the mixed esters described later, for us. We wish to express our indebtedness to them for these results, from which certain important conclusions are possible.

TABLE IV  
*Composition of the  $C_{18}$  Esters as Evaluated by Different Methods*

	Crystallization	Method Used Thiocyanometric per cent	Spectroscopic*
Stearate	11.6	10.2	11.9
Octadecenoate	68.3	70.5	68.9
Octadecadienoate	20.1	19.3	18.1
Octadecatrienoate	—	—	0.8
Octadecatetrenoate	—	—	0.3**

\* Results of three determinations by Kraybill and Beadle.

\*\* Calculated as arachidonate, but recorded here as octadecatetrenoate.

In Table IV there are recorded the compositions of the  $C_{18}$  esters as calculated from the direct crystallization and thiocyanometric data, which calculations do not take into account trienoic and tetrenoic esters, in comparison with the results obtained by Kraybill and Beadle. For reasons which follow later, the unsaturated esters in this table are named non-specifically, rather than as oleate, etc.

\* This test is sensitive for esters of the arachidonate type, but not nearly so with methyl linolenate, the hexabromide of which is more soluble.

The thiocyanometric results in Table IV show up rather poorly for two reasons: (1) because stearate is determined by difference, and (2) because the octadecenoate and octadecadienoate results are thrown into error since they do not take into account the small amounts of more highly unsaturated esters present. Values for stearate and octadecenoate agree well by crystallization and spectroscopically. Also, by crystallization the dienoate value includes two, three and four double bond esters, and agrees well with the sum of these three esters as determined spectroscopically.

The procedure in Chart 4 successfully isolated methyl stearate of about 95% purity ( $C_{18}$ , 12.2 g.), methyl octadecenoate ( $C_{18}$ , 54.3 g.) and a number of filtrate fractions containing dienoate, and more unsaturated esters ( $F_3$ - $F_5$ , 31 g.). In view of the previous finding of Millican and Brown (13) of octadecenoic acids other than oleic acid in a number of lipids of animal origin, including human body fat, lard and beef fat, the methyl octadecenoate above was converted into the free acid (Iodine No., 89.2) and repeatedly crystallized as described in Chart 5.

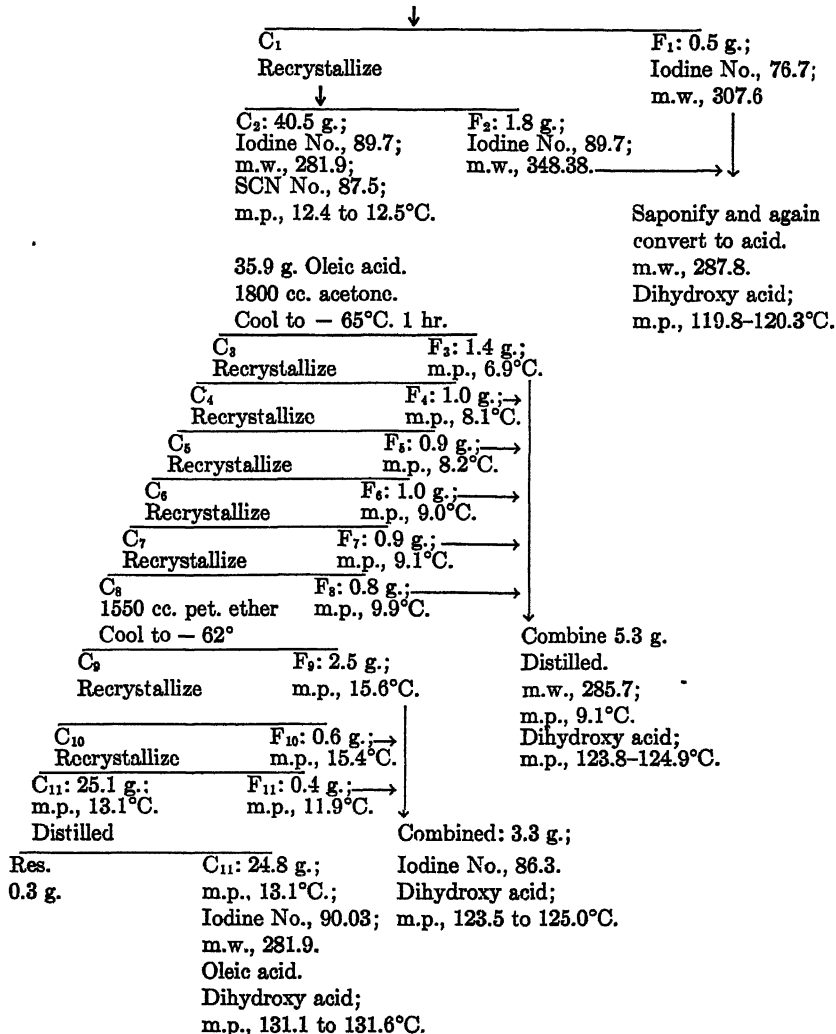
Fraction  $C_{11}$  of Chart 5 is oleic acid of high purity, as is evidenced by its melting point, iodine number, molecular weight and melting point of the dihydroxy acid. Nearly 60% of the original mixture of octadecenoic acids was recovered as oleic acid. The several filtrate fractions, on the other hand, with the exception of  $F_9$  and  $F_{10}$  which may have been the higher melting modification of oleic acid, gave melting points too low for oleic acid or for mixtures of oleic and stearic acids. For reasons summarized by Millican and Brown (13) the data in Chart 5 are strongly indicative of the presence of one or more isomeric octadecenoic acids in human milk fat. With the exceptions noted, the melting points of the filtrate fractions rose from 6.9°C. in  $F_3$  to 11.9°C. in  $F_{11}$ , which is an indication that each successive filtrate fraction is poorer in the isomeric octadecenoic acids.

Our next objective was to study further the linoleate concentrates of Chart 4. Filtrates  $F_3$  and  $F_4$  of this chart, containing most of the linoleate, were combined, and the free acids were prepared: Iodine No., 152.5; m.w., 279.7; ether-insol. bromides, trace; tetrabromide no. (Tb. No.), 29.9. From the iodine number, and calculated as a binary mixture, these acids are composed of 32% octadecenoic and 68% octadecadienoic acids. On the basis of a theoretical tetrabromide number of 102.9 for pure linoleic acid (14), the mixture contains about

Chart 5

*Crystallization Study of Octadecenoic Acids*

42.7 g. in 2 liters petroleum ether

Cool to  $-65^{\circ}\text{C}.$  for 1 hour

29% of linoleic acid. By difference, the remainder of the mixture (39%) is composed of isomeric octadecadienoic acids, which do not

yield the usual petroleum ether-insoluble tetrabromides. Thus, about 43% of the dienoic acids present is linoleic acid. It is to be noted that these calculations are only approximations, in part because of the spectroscopic demonstration of trienoic and tetrenoic acids in this fraction. The presence of both linoleic and isomeric octadecadienoic acids in this mixture is further borne out by analyses of the several sub-fractions in Chart 6.

### Chart 6

#### *Crystallization of $F_3$ and $F_4$ (Chart 4)*

19.7 g. acids (Iodine No., 152.5) in 500 cc. acetone. Cool to $-70^{\circ}\text{C}$ . Warm to $-54^{\circ}\text{C}$ . and filter		
↓		
$C_1$ : 6.4 g.; Iodine No., 141.5;* Tb. No., 17.5; m.p. bromide	$F_1$	Cooled to $-75^{\circ}\text{C}$ ., no ppt. 250 cc. acetone removed. Cooled to $-75^{\circ}\text{C}$ .
↓		
112.2–113.7°C.	$C_2$ : 9.6 g.; Iodine No., 158.3.* Add 250 cc. petroleum ether. Cool to $-68^{\circ}\text{C}$ .	$F_2$ : 3.6 g.; Iodine No., 155.5;* Liquid at $-20^{\circ}\text{C}$ .; Tb. No., 22.0
↓		
	$C_3$ : 3.2 g.; Iodine No., 162.9;* Tb. No., 47.4.	$F_3$ : 5.5 g.; Iodine No., 154.9;* Tb. No., 45.2.

\* Calculated as binary mixtures of octadecenoic and octadecadienoic acids, iodine numbers show  $C_1$  to contain 57 (17) %,  $C_2$  74%,  $C_3$  80 (47) %,  $F_2$  71 (22) % and  $F_3$  71 (45) % octadecadienoic acid. Values in parenthesis are approximate linoleic acid contents, calculated from the tetrabromide numbers.

The identification of the trienoic and tetrenoic esters, shown spectroscopically to be present in the  $C_{18}$  esters, was next attempted. The original esters of this fraction gave no ether-insoluble bromides. Further, the filtrate acids, crystallized in Chart 6, and representing a four-fold concentration of the more unsaturated esters, yielded only a trace of polybromoacids. Miss Frechtling's results, mentioned previously, showed that 25 mg. of linolenic acid in 2 g. of a fatty acid mixture can be detected by this method; and that 3 mg. of methyl

arachidonate (and presumably arachidonic acid) will yield appreciable amounts of octabromide. These values, however, represent about the limit of sensitivity of the method. Thus the concentrate noted above contained less than these minimum amounts of either linolenic or arachidonic (tetrenoic) acids. The bromide test was not applied to the sub-fractions obtained in Chart 6. However, a specimen of acids, similar to those used in Chart 6, was prepared from another batch (131 g.) of  $C_{18}$  esters and, by crystallization from acetone, a concentrate of filtrate acids was prepared in which the polyenic acids had been concentrated about nine times. A 2 g. specimen of this concentrate yielded 12.1 mg. of ether-insoluble bromide, which, by interpolation from our linolenic acid-hexabromide curve, would amount to 51 mg. of linolenic acid; calculated to the original  $C_{18}$  esters, this corresponds to a content of 0.3% of linolenic acid, whereas spectroscopically 0.8% of trienoic acid was found. However, the melting point behavior of the bromide (darkened and sintered without melting at  $180^{\circ}$ – $230^{\circ}$ ) was definitely not that of hexabromostearic acid. We feel, therefore, that little or no linolenic acid occurs in human milk fat but, rather, an isolinolenic (octadecatetrienoic) acid which does not yield the usual hexabromide melting at  $180^{\circ}\text{C}$ .

The question of the nature of the tetrenoic acid in the  $C_{18}$  esters is more difficult to answer. Methyl arachidonate is the lowest boiling of the  $C_{20}$  esters, and could conceivably be found in the  $C_{18}$  main fraction as a contaminant of this fraction. However, the presence of arachidonate in this material does not seem likely in view of the known efficiency of the column used in the distillation. If the ester were methyl arachidonate, the 12.1 mg. of ether insoluble bromide, mentioned previously, can be interpolated as 21.4 mg. methyl arachidonate in the sample brominated, or about 0.1% of the original  $C_{18}$  esters. The spectrophotometric finding was 0.3% of tetrenoate in these esters. These data are inconclusive, because the interpolation curve was based on bromination of methyl arachidonate and not on arachidonic acid as in the present instance. Evidence in favor of the view that the tetrenoic acid is actually an octadecatetrenoic acid was obtained as follows. It occurred to us that if the tetrenoate of the  $C_{18}$  main fraction was of the  $C_{20}$  series, we would find less of it in the  $C_{16}$ – $C_{18}$  intermediate ester fraction than in the  $C_{18}$  fraction, while, on the other hand, if it were an octadecatetrenoate, since this would be the lowest boiling of the  $C_{18}$  esters, a somewhat higher concentration

of this material would be found in this intermediate fraction. Consequently, the  $C_{16}$ - $C_{18}$  fractions from the two main distillations were combined, the free acids prepared, and these were crystallized once from acetone. The filtrate acids thus obtained represented a concentration of polyenic acids of about three times; they gave a polybromide number of 0.2; the bromides again failed to melt at  $180^\circ$ , in fact remained a dark powder up to  $250^\circ$ . In view of the fact that this intermediate fraction employed as a starting material in this experiment contains about 35% of  $C_{16}$  esters, actually the  $C_{18}$  component is nearly twice as rich in tetrenoate as the  $C_{18}$  main fraction. We believe that this supports the view that an octadecatetrenoic acid occurs in this specimen of fat. Since the original esters were shown spectroscopically to contain 1.0% of tetrenoate, a result substantiated by the yield of bromides, we have apportioned this in Table I as 0.2% octadecatetrenoate and 0.8% arachidonate.

The presence of octadecatetrenoic acid (in the early literature this was called *clupanodonic acid*) in a milk fat has not been previously described. This acid occurs in fish oils as a normal constituent, and it is possible that its origin in this specimen is dietary, since it is known that dietary fatty acids appear readily in milk fat.

#### *The Nature of the Fatty Acids of Carbon Series Above $C_{18}$*

Still-pot and column hold-up esters from the two main distillations amounted to about 43 and 32 g., respectively. In both instances, these esters, after analysis, were further fractionated, but the efficiency of the column used was poor, so that main fractions were not obtained. By the use of the arachidonate-polybromide curve, mentioned previously, these esters were shown to contain 1.7 and 2.5 g. of methyl arachidonate respectively, or, based on the original esters, 0.4 and 0.7%. Since an additional 0.2% of tetrenoic esters was found in the  $C_{18}$  esters, the total recovery of tetrenoic esters was 0.6 and 0.9% in the two distillations, as compared to 1.0% found by direct methods on the original esters. The difference here may be the result of loss of this type of ester by pyrolysis, etc., during the prolonged distillation, the loss amounting to 0.4 and 0.1% in the two instances.

Crystallization studies were carried out on several of the  $R_1$  sub-fractions and on the  $R_2$  fraction of Table II. In no case was more than 15% of saturated acids recovered, and these were all mixtures. Assuming  $R_1$  to contain this amount of saturated esters and also about

4% arachidonate, the remaining esters by calculation will have an iodine number of about 100 which is considerably above that of monoethenoic esters of the  $C_{20}$ - $C_{24}$  series. Thus, appreciable amounts of dienoic or trienoic (or both) esters must be present in these series. At this point it occurred to us that valuable information concerning the nature of these acids could be obtained from a comparison of spectroscopic analysis of the  $C_{18}$  esters with a similar analysis of the original mixed esters. These results, together with data on the original fat, are shown in Table V.

TABLE V

*Spectroscopic Analysis of Human Milk Fat and Its Mixed Methyl Esters, in Comparison with Values Found in the  $C_{18}$  Fraction*

	Spectroscopic Analysis			Amounts Recovered Below $C_{18}$ <sup>†</sup>	Total $C_{18}$ and Below	Total Above $C_{18}$ <sup>†*</sup>
	Fat	Mixed Esters	$C_{18}$ Esters			
			<i>per cent</i>			
Monoethenoic	39.8	41.1	39.3	3.2	42.5	—
Dienoic	12.6	12.2	10.3	0	10.3	1.9
Trienoic	0.8	0.8	0.5	0	0.5	0.3
Tetrenoic	1.1	1.0	0.2	0	0.2	0.8
Saturated	45.8	44.9	6.8	34.8	41.6	3.3

<sup>†</sup> From Table I.

<sup>†\*</sup> By subtracting data in preceding column from values found on mixed esters.

In Table V by subtracting the total amount of esters recovered in the  $C_{18}$  and lower fractions from the results found by spectroscopic analysis of the mixed esters it is possible to estimate the approximate amounts of various types of esters above  $C_{18}$ . Two inconsistencies are immediately apparent. More monoethenoic acids (1.4%) were recovered in the  $C_{18}$  and lower esters than are found in the original esters. Further, differences in the two series of data show 3.3% of saturated acids to be in series above  $C_{18}$ , which is considerably more than crystallization data on the higher fractions seem to warrant. Further, the  $C_{20}$  and above material contained 1.9% of dienoic esters, 0.3% trienoic and, as noted previously, 0.8% of arachidonate. Baldwin and Longenecker reported strong evidence for the presence of high molecular weight dienoic acids, based on spectroscopic examination of



their higher fractions. We feel that our evidence, although indirect, equally supports the occurrence of trienoic acids in these higher series, since the alternative view that 0.3% of trienoic material was destroyed during the distillation does not seem likely to be correct.

Further inquiry into the occurrence of octadecatetrenoic and of high molecular weight dienoic and trienoic acids in human milk fat and into their possible occurrence in butter fat will be of considerable interest, both from the chemical and nutritional point of view.

### SUMMARY

A large specimen of human milk fat has been examined quantitatively with reference to the amounts and nature of its component fatty acids. No evidence could be found for the presence of more than traces of acids below  $C_{10}$ . As a result of distillation of two specimens of methyl esters, prepared from this fat, and applying low temperature crystallization technics to the main fractions, a number of pure esters and acids have been isolated for the first time and identified. The presence of monoethenoic acids from  $C_{10}$  to  $C_{18}$  has been verified; the  $C_{18}$  acids of this type were shown to be made up chiefly of oleic acid, together with isomeric octadecenoic acids. About 40–50% of the octadecadienoic acids was shown to be ordinary linoleic acid, the remainder being presumably the isolinoleic acid found in butter fat. Small amounts of both trienoic and tetrenoic acids of the  $C_{18}$  series were also shown to be present, although the amount of linolenic acid is negligible. The finding of dienoic acids of series above  $C_{18}$ , reported by Baldwin and Longenecker, was confirmed, and in addition evidence was presented for the presence of high molecular weight trienoic acids. This specimen of fat is much more similar to human body fat than it is to a typical milk fat.

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# **The Effect of Advancing Age on Dietary Thiamine Requirements**

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## **INTRODUCTION**

In previous articles (1, 2, 3) we have presented evidence of need for a heightened dietary percentage of thiamine when experimental animals are kept in an environment of tropical warmth. The need for this dietary enrichment seemed to be due mainly to a smaller food intake, and not to an increase in absolute daily requirement. This suggested that the need for thiamine depended more upon the mass of metabolizing tissue in the animal than upon caloric intake. Thus the deduction was drawn that diets for hot weather or tropical climates should be richer in thiamine than those needed for optimal nutrition in temperate coolness.

Cowgill (4) presented extensive data pointing to the conclusion that thiamine requirement bore practically a straight line relationship to body weight, although he failed to stress the point that diets of adequate vitamin content for young animals would thereby become inadequate for older individuals with more sluggish tissue combustion and reduced appetite. Many of the more recent workers in nutrition (5) have held that thiamine requirement is most closely related to caloric intake, and they thus speak of a definite thiamine/caloric ratio.

Whether thiamine requirement (and possibly that of other B-vitamins) is based upon caloric intake or upon the mass of metabolizing tissue becomes a very important question in practical dietetics when one comes to consider the reduced food intake of ill health or advancing age. We wish now to report a sharp rise in dietary thiamine concentration needed for optimal growth response with advancing age. Twelve-month-old rats at 450 to 500 g. body weight eat only slightly more

food than 2-month-old ones at 180 g., and for optimal growth they require a diet almost twice as rich in thiamine.

White rats (Sprague-Dawley males) were purchased as weanlings and divided between the hot room (90–91°F. and 60–70% relative humidity) and one kept at 68–70°F. The synthetic diets used were the ones previously described as optimal for tropical and temperate environments (3), with graded differences in thiamine content as indicated in Table I. Weekly weight changes were observed while

TABLE I

*Dietary Thiamine Concentration Needed for Optimal Rat Growth with Advancing Age*

Room Conditions	68°F				90–91°F and 60–70% relative humidity					
	0.8	1.2	1.6	2.0	0.8	1.2	1.6	2.0	2.5	3.0
Dietary Thiamine (mg./kg. of diet)										
<i>1st test: completed at 2 mo.</i>										
Wt. gain (g.), last 3 of 5 wks. on diets	{ +39.5 ± 3.6	{ +80.1 ± 1.1	{ +73.7 ± 2.2†		{ -3.6 ± 0.4	{ +17.5 ± 0.9	{ ± 65.1 ± 1.3			
Final av. wt. (g.)	132 (8)*	174 (8)	170 (8)		78 (9)	100 (9)	138 (9)			
Av. food intake (g./rat/week)	90	93	100		43	52	65			
Thiamine intake (γ/rat/day)	10	16	23		5	9	15			
<i>2nd test: completed at 7 mo.</i>										
Wt. gain (g.), last 2 of 5 wks. on diets	{ - 3.0 ± 0.8	{ +12.3 ± 1.1	{ +11.6 ± 1.1		{ - 8.0 ± 1.1	{ + 7.6 ± 1.7	{ +21.3 ± 2.2			
Final av. wt. (g.)	361 (8)	380 (8)	374 (8)		304 (9)	323 (9)	356 (9)			
<i>3rd test: completed at 18 mo.</i>										
Wt. gain (g.), last 3 of 8 wks. on diets	{ -24.8 ± 3.0	{ + 6.1 ± 1.1	{ +14.7 ± 1.8			{ - 3.0 ± 1.8	{ +13.6 ± 2.7	{ +13.7 ± 1.1	{ ± 16.2 ± 1.8	
Final av. wt. (g.)	414 (8)	422 (7)	444 (7)			409 (8)	419 (7)	424 (7)	412 (7)	
Av. food intake (g./rat/week)			133					85		
Thiamine intake (γ/rat/day)			30					30		
<i>4th test: completed at 21 mo</i>										
Wt. gain (g.), last 3 of 5 weeks on diets		{ -89.0 ± 3.5	{ -50.0 ± 7.2	{ -5.0 ± 4.2			{ -90.0 ± 5.0	{ -57.0 ± 5.1	{ ± 0.3 ± 5.1	{ ± 0.3 ± 4.0
Final av. wt. (g.)		470 (5)	487 (6)	522 (6)			400 (4)	435 (5)	458 (5)	

\* Numbers of rats per group are indicated in parentheses.

† Probable errors of the mean values given.

feeding these diets *ad libitum* for a 5-week period. The rats were then placed on a commercial mixture\* as the sole food for 4 months, all those of each room being mixed together in one large cage for this interim. At 6 months of age they were again divided into groups of approximately equal weight and again kept for 5 weeks on the graded synthetic diets. This was again followed by a 3-month period on chow and by 8 weeks on the test diets at 10 months of age.

Table I presents the summated weight changes exhibited by the

\* Purina dog chow biscuits.

various groups during the final weeks of each test period. During the first test period, which the rats began as weanlings, it is obvious that 0.8 mg./kg. of dietary thiamine was mildly inadequate in the cold and sharply so in the heat; 1.2 mg./kg. was completely adequate in the cold and mildly inadequate in the heat; 1.6 mg./kg. was still slightly inadequate for the hot-room rats. So we may speak of 0.8 mg./kg. as the inadequacy threshold for weanling rats in the cold and 1.6 mg./kg. in the heat.

At 7 months of age the cold-room rats showed sharp inadequacy at 0.8 mg./kg. but optimal growth at 1.2 mg./kg.; those in the heat exhibited sharp inadequacy now at 1.2 mg./kg., mild inadequacy at 1.6 mg./kg., but optimal response at 2.0 mg./kg. At 12 months of age 1.2 mg./kg. had become insufficient for optimal growth in the

TABLE II  
*Previous Records of Food and Thiamine Intake Producing Optimal  
Rat Growth in Heat and Cold*

Average weight of rats at 8 weeks	Food eaten g./rat/week		Thiamine intake γ/rat/day		Reference
	At 68°F.	At 90-91°F.	At 68°F.	At 90-91°F.	
180	105	75	15	21	(6)
180	100	72	14	21	(3)
180	95	67	14	19	(7)
—	—	—	—	—	
180	100	71	14	20	Averages

cold and 1.6 mg./kg. sharply insufficient in the heat, optimal growth then requiring slightly over 1.2 mg./kg. and 2.0 mg./kg., respectively. When tested as old rats at 21 months of age, after several of the group had died, it was found that 2.0 mg./kg. in the cold and 3.0 mg./kg. in the heat were necessary to halt the weight loss then generally in progress.

It is unfortunate that the diet series used for the first testing in the heat did not include a higher thiamine level which would have given optimal growth. In three recent reports (6, 3, 7), as summarized in Table II, we have shown that growth on our synthetic diets is optimal at a thiamine concentration of 1 mg./kg. in the cold and at 2 mg./kg. in the heat. At these levels, Sprague-Dawley rats at eight weeks of age weigh close to 180 g. in both heat and cold. Those in the cold eat, on the average, 100 g. of food per week at that age, while those in the

heat consume about 70 g. This means a daily thiamine intake per 180 g. rat of 14  $\gamma$  in the cold and 20  $\gamma$  in the heat.

Food consumption was not estimated during all the test periods of this study, but it was done for one group in each room showing optimal growth at 12 months of age. These rats, weighing somewhat over 400 g., were eating only slightly more food than was consumed at optimal thiamine levels by the 180 g. rats at 8 weeks of age. However, the daily thiamine intake needed for this optimal growth had risen to 30  $\gamma$ . Tested again soon afterward, at 14 months of age (Table III), the daily thiamine intake was found to be 25  $\gamma$  for the cold-room

TABLE III

*Dietary Percentage of Protein Required for Optimal Growth in 14-Month-Old Rats*

Dietary protein per cent	At 65°F.			At 90-91°F., 60-70% Rel. Hum.		
	18	24	30	18	24	30
Wt. gain (g.), last 6 of 8 weeks on diets	{ 32.0 $\pm 1.3$	{ 32.0 $\pm 0.9$	{ 16.4 $\pm 4.4$	{ 32.0 $\pm 2.3$	{ 27.5 $\pm 4.2$	{ 17.0 $\pm 1.3$
Final av. wt. (g.)	533 (7)*	514 (7)	510 (7)	459 (10)	458 (8)	456 (8)
Aver. food intake (g./rat/week)	117	128	124	85	83	75
Thiamine intake ( $\gamma$ /rat/day)	25	27	27	30	30	27

\* Number of rats per group indicated in parentheses.

rats and 30  $\gamma$  in the heat. It is thus obvious that daily thiamine requirement (in  $\gamma$  per rat) rises sharply with advancing age and increasing body size, even though food consumption shows little change.

As weanlings in the heat, these rats go into acute thiamine deficiency (and die) on 0.4 mg./kg. or less of dietary thiamine. At 11 months they do so at 1.0 mg./kg. but not at 1.2 mg./kg. At 18 months of age acute and fatal thiamine deficiency developed on diets containing 1.5 mg./kg. but not at 1.6 mg./kg.

It seems quite likely, therefore, that thiamine requirement (with *ad libitum* feeding) is proportional to the mass of metabolizing tissue and largely independent of daily caloric intake. In cool surroundings a larger food intake permits the use of diets with fewer milligrams of thiamine per kilo of food. This observation becomes very important

with the loss of appetite and reduced food consumption of advancing age or ill health. It would also seem to indicate a need for vitamin supplementation when weight-reduction diets are used.

Indicated in Table III are the mean weight gains at various levels of dietary protein in heat and cold when fed at 14 months of age. Optimal weight gain still took place at 18% dietary protein just as it had when the rats were of weanling age. The diets used here had thiamine contents of 1.5 mg./kg. in the cold and 2.5 mg./kg. in the heat, as these amounts were indicated by the results set forth in Table I as approximately optimal at 14 months of age.

One effort to test for optimal choline requirement was interrupted by a heat wave which interfered with satisfactory temperature control in the hot room, but incomplete results indicated optimal growth in the cold at 0.4 g./kg. and in the heat still at 5.0 g./kg. Growth and food consumption were definitely retarded in the cold at 1.5 g./kg.; while in the heat even 7.0 g./kg. was well tolerated. It would thus seem that perhaps choline is tolerated less well with advancing age in the cold, but fully as well or better in the heat.

## DISCUSSION

These findings suggest the advisability of investigating age changes in requirement (in mg./kg. of diet) for the other B-vitamins. There would already seem to be clear indication for thiamine supplementation in diets for the aged or ill individuals whose total food intake is reduced. It is of little importance to such people that their absolute requirement for thiamine (*in mg. per day*) may remain unchanged, if their intake of the vitamin-containing food is to be sharply curtailed by ill health or the falling tissue metabolism of advancing age.

Since the publication of our first paper on heightened thiamine requirement (*in mg. per kg. of diet*) for animals adapted to tropical heat (1), numerous articles have been written in attempts to disprove our findings. In several instances (8) these studies were carried out on men or animals subjected to only brief exposure to tropical heat (less than 2 weeks), and hence the results could not reasonably be applied to tropical populations (9). In many other cases (10) investigators claimed to have refuted our findings because they found no significant difference in *absolute* requirement (*in mg. per day*), overlooking entirely our continued insistence that our results dealt only



with vitamin concentrations in the diet (mg. per kg. of diet). One review journal has repeatedly persisted in this confusing attitude (11).

In our studies we purposely incorporated the vitamins into the diet mixtures, since that most nearly simulates the dietary problems people face. What an individual's absolute vitamin requirement may be is of little importance to him if he is unable to consume enough of his given diet to meet these requirements. It has been clearly demonstrated that diets of adequate vitamin composition for animals adapted to temperate coolness become sharply insufficient for those kept in tropical warmth—merely because those in the heat fail to ingest a sufficient amount of the food. The findings here presented demonstrate that the same holds for the reduced food consumption of advancing age.

### CONCLUSIONS

Thiamine requirement per g. of food rises sharply with advancing age in rats adapted to tropical warmth or temperate coolness while the absolute requirement in  $\gamma$  per day advances with increasing body size. A similar rise in dietary thiamine concentration was found necessary to prevent signs of acute polyneuritis and death with advancing age and increasing body weight. At 18 months of age rats kept in tropical heat develop acute and fatal thiamine deficiency at dietary thiamine concentrations adequate to support optimal growth in temperate coolness.

Thiamine requirement, therefore, seems largely conditioned by the mass of metabolizing tissue and relatively independent of caloric intake under conditions of voluntary *ad libitum* feeding.

Protein and choline requirements per kg. of food seem to change little with the reduced food intake per unit of body mass in advancing age.

The conclusion seems justified that diets for the aged or those in ill health should be thiamine-enriched to compensate for reduction in amount of food consumed. It seems probable that the same principle should be applied with the sharp caloric restriction of weight-reducing and hot weather diets.

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# Method for the Direct Determination of Diacetyl in Tissue and Bacterial Filtrates

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## INTRODUCTION

Many methods for the determination of diacetyl and acetoin have been devised which are applicable to various types of investigation.

Pien *et al.* (1), developed a colorimetric method for the determination of diacetyl with certain *o*-diaminobenzene derivatives which, in the presence of strong acids, give compounds having a yellow color. They first used 3,4-diaminotoluene but later (2) found that diaminobenzidine gave a stronger color. Stahly and Werkman (3) found that the quantitative measurement of the nickel dimethylglyoxime procedure first proposed by Lemoigne (4) and modified by van Niel (5) could be applied to fermentation distillates. This particular method is useful for determining only relatively large amounts of diacetyl. Prill and Hammer (6) devised a colorimetric procedure for the microdetermination of diacetyl in butter and milk based on the formation of colored ammonioferrous dimethylglyoximate. This method, while apparently sensitive and accurate, is not readily applicable to metabolic studies as both pyruvic acid and methylglyoxal produce a similar color with the reagents. Stotz and Rahorg (7) have described a colorimetric determination employing the precipitate of nickel dimethylglyoxime. The precipitate is oxidized with bromine and the nickel determined by the formation of a soluble colored complex with excess dimethylglyoxime. The method, though sensitive and rather accurate in determining amounts as low as 10  $\gamma$  in tissues, is time-consuming, since at least 12 to 14 hours are required for each assay.

None of the above procedures is applicable to tissue filtrates directly, as the diacetyl must first be distilled before assay. It was desirable to devise a rapid, accurate method which could be applied directly to bacterial or tissue filtrates.

The method here described is based on the condensation of diacetyloxime with urea as first described by Fearon (8). Archibald (9) has recently described a modification of Fearon's method for the determination of citrulline which gives a reaction apparently similar

to that of urea. We have adapted the converse of the modified method of Archibald to the determination of diacetyl and acetoin using urea in place of citrulline since the former is readily available.

### EXPERIMENTAL

*Principle:* The method depends on the production of a colored compound by the reaction of diacetyloxime and urea in the presence

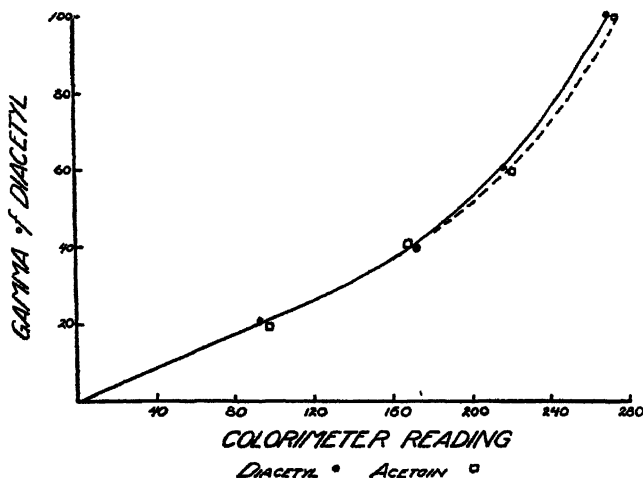


FIG. 1

Standard Curve of Diacetyl Showing Recovery of an Equal Amount of Crystalline Acetoin by  $\text{FeCl}_3$  Oxidation

of strong acid. The colored compound has not been identified but may be some quinoxaline-like derivative.

*Reagents: Diacetyl Standard*—A solution of 100 mg. of diacetyl in 100 ml. of  $\text{H}_2\text{O}$ . This solution should be kept in the ice box when not in use and, due to the volatility of diacetyl, should be freshly prepared about every two weeks. For assay this solution is diluted 1:10 to give a concentration of 100  $\gamma$  per ml.

*Urea Solution*—3% solution in water

*Hydroxylamine*—a solution containing 10 mg. per ml. in water

*Sulfuric-Phosphoric Acid Mixture*—1 volume of conc.  $\text{H}_2\text{SO}_4$  and 3 volumes of syrupy  $\text{H}_3\text{PO}_4$

*Procedure:* Various dilutions of a trichloroacetic or metaphosphoric acid filtrate of the tissue or bacterial juice are made in such a manner that 1 ml. contains approxi-

mately 100  $\gamma$  or less of diacetyl. One ml. of the dilution is then transferred to a test tube, and 1 ml. of the hydroxylamine solution, 1 ml. of urea solution and 2 ml. of the  $\text{H}_2\text{SO}_4\text{-H}_3\text{PO}_4$  mixture are added. The volume is then adjusted to 6 ml. with water and the tube is rotated rapidly to mix. A diacetyl standard is run with concentrations of 100, 80, 60, 40, and 20  $\gamma$  of diacetyl treated in the same manner as the filtrates above.

After mixing, the tubes are placed in a boiling-water bath for 35–40 minutes, cooled, and the optical density measured on a photometer using a 470  $\text{m}\mu$  filter. A reagent blank is used to set the instrument scale at zero. Throughout this work a Klett-Summerson photoelectric colorimeter was used. A standard curve is plotted from the known diacetyl readings and the diacetyl (or acetoin) concentration of the

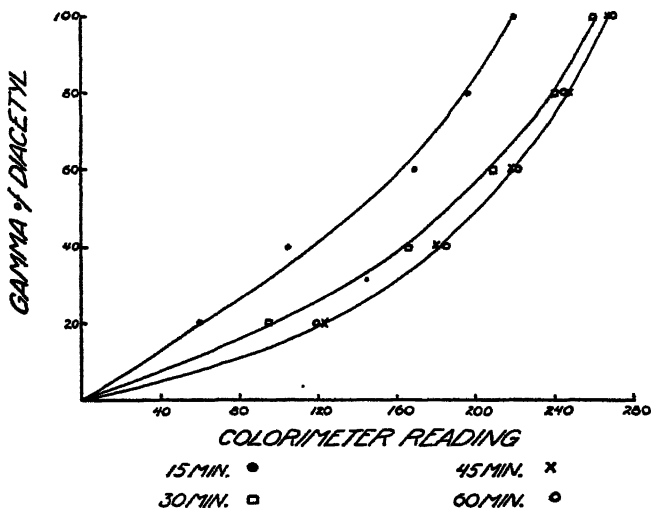


FIG. 2

Curves of Diacetyl Standard at Varying Lengths of Time in Boiling  $\text{H}_2\text{O}$  Bath

unknown calculated by interpolation. Fig. 1 shows a typical standard curve for diacetyl and the curve of a similar amount of crystalline acetoin determined at the same time by oxidation and distillation. Though the intensity of the color is not exactly proportional to concentration, the curve can be reproduced regularly with considerable accuracy.

*Effect of Time of Heating on Color Intensity:* Fig. 2 shows the effect of heating on the increase in the intensity of the color formed by known quantities of diacetyl. After 35 minutes no increase occurs, hence this time has been used as the minimum for heating. With shorter times variations have been found in the intensity of the color which affect the determination of the concentration of diacetyl.

*Recovery of Diacetyl from Tissue Filtrates:* To determine whether interfering substances were present, 50 g. of fresh liver were ground to a paste with 50 ml. of water in a Waring blender. To 1 g. samples of the paste, 1 mg. of diacetyl was added, the volume made to 5 ml. with water and the mixture thoroughly shaken. Two ml. of 10% trichloroacetic acid or 2 ml. of 10% metaphosphoric acid were then added, the tube shaken and the resulting precipitate removed by centrifugation. The precipitate was washed twice with 1 ml. portions of water and the volume of the filtrate made to 10 ml. One ml. of the resulting filtrate should contain, therefore, an excess of 100  $\gamma$  of diacetyl if the tissue contained any substance producing a color with

TABLE I  
*Recovery of Added Diacetyl from Tissue*

Diacetyl	Liver paste	Colorimeter reading	Diacetyl	Per cent recovery
$\gamma$			$\gamma$	
0		0		
100		268		
80		242		
60		220		
40		175		
20		110		
100	1 g.	261	94	94
50	1 g.	195	46	92
25	1 g.	133	24	96
	1 g.	0	0	

Liver precipitated with 2 ml. of 10% metaphosphoric acid. Heated for 35 minutes.

the reagents. A like amount of liver paste was treated in a similar manner and both assayed at various concentrations according to the procedure outlined. The results of the recovery experiments are shown in Table 1. The recovery shows fairly good agreement between 20  $\gamma$  and 100  $\gamma$  and indicates that there is no substance present in liver at this concentration which markedly interferes with the recovery of diacetyl.

*Determination and Recovery of Added Acetoin:* Acetoin, which does not itself give a color with these reagents, can be determined by the procedure described if first converted to diacetyl by oxidation. The method of Stotz and Raborg (7), using  $\text{FeCl}_3$  and  $\text{H}_2\text{SO}_4$ , was found satisfactory for the oxidation of small amounts of acetoin.

To 1 g. of the liver paste 1 ml. of a solution of 100 mg. of crystalline acetoin diluted to 100 ml. was added. The mixture was made to 5 ml. with water, shaken and precipitated with 2 ml. of either 10% trichloroacetic acid or 10% metaphosphoric acid. The precipitate was washed twice with 1 ml. volumes of water and the filtrate and washings oxidized (7, 10), with the exception that the distillate was delivered into a 10 ml. graduated test tube containing 2 ml. of the 10 mg. per ml. hydroxylamine solution. The distillate was collected to the 10 ml. mark. Aliquots of this solution of diacetyloxime were then assayed and the percentage recovery determined. As acetoin is converted

TABLE II  
*Recovery of Added Acetylmethylcarbinol from Liver Paste*

Diacetyl $\gamma$	A.M.C. $\gamma$	Liver paste	Colorimeter reading	A.M.C. recovered $\gamma$	Percentage recovery
0			0		
100			272		
80			250		
60			224		
40			179		
20			103		
	100	1 g.	266	95	95
	60	1 g.	230	63	105
	40	1 g.	185	42	105
	30	1 g.	161	32	106
		1 g.	0	0	

Liver precipitated with 2 ml. of 10% metaphosphoric acid. Heating time 35 minutes.

quantitatively to diacetyl by this procedure our results are calculated as acetoin. The weight relationship is not exactly equivalent but, since the ratio between the weights of acetoin and diacetyl is 1.02, the discrepancy in a 100  $\gamma$  amount is negligible.

Controls using liver paste without added acetoin were used as checks on the recovery experiments.

Typical data for the recovery of acetoin by this procedure are given in Table 2. All recoveries in the lower concentrations ran somewhat high. Since the liver paste alone showed no acetoin present, the results cannot be ascribed to the paste. However, the method appears to be accurate to within  $\pm 10\%$  with amounts as low as 25  $\gamma$ .



*Effect of Light on the Color Reaction:* Contrary to the findings of Fearon and Archibald, light was found to have little effect on the stability of the color produced by the reaction up to at least four hours after boiling. One set of standard diacetyl tubes was exposed in a beaker to bright daylight for a period of 6 hours. Readings every 2 hours showed little, if any, change in the optical density. However, reading of the tubes after 4 hours is not recommended.

*Specificity of the Method:* Pyruvate, acetaldehyde, lactate, glucose, 2,3-butylene glycol or unoxidized acetoin in 1 mg. amounts do not react sufficiently with the reagents to interfere with the recovery of diacetyl. Acetoin, since it is readily oxidized to diacetyl, must be determined separately or on another aliquot of the filtrate, in which case the acetoin present can be calculated as the difference between the oxidized and unoxidized samples.

#### SUMMARY

A sensitive, reasonably rapid method for the determination of diacetyl and acetoin is presented.

Diacetyl can be determined directly on tissue and bacterial filtrates without previous distillation.

Acetoin is determined by oxidation to diacetyl by  $\text{FeCl}_3$  in acid solution followed by distillation.

The method appears to be sensitive for diacetyl and acetoin in concentrations at least as low as 25  $\gamma$  with an accuracy of  $\pm 10\%$ .

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# Preparation of Samples for the Microbiological Assay of Pantothenic Acid \*

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## INTRODUCTION

Although numerous methods have been proposed for the microbiological determination of pantothenic acid (1, 3-12, 14), relatively little work on the preliminary treatment of the sample has been reported. However, some sort of hydrolytic procedure must be applied if the total pantothenic acid content is to be measured, because it is well known that this vitamin exists in tissues to a large extent in combined forms which are not utilizable by the test organisms employed (7, 8, 11, 15).

Since both acid and alkaline hydrolysis are excluded by the lability of pantothenic acid, and autolysis in many cases is not feasible, the use of hydrolytic enzymes remains as the only practicable method. Enzymatic digestion of the sample, however, necessitates a correction for the pantothenic acid content of the enzyme preparation used, and entails a danger that some of the vitamin may be destroyed or that substances which interfere with the assay may be liberated. The latter effect has been particularly troublesome in earlier work and has probably been responsible for at least part of the increases in apparent pantothenic acid content observed after enzyme treatment of certain materials (7).

It was the purpose of the present investigation to repeat and extend

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previous studies on the effect of digesting pure pantothenic acid and representative biological materials with various enzymes, making every effort to avoid errors from interfering substances liberated during the digestion. The factors particularly studied were the kind of enzyme, its concentration in the digestion mixture and the time and temperature of incubation.

### EXPERIMENTAL RESULTS

The method of assay used throughout was that of Neal and Strong (7) as modified by Ives *et al.* (4). All assays were run in duplicate on different days. Before being assayed all digests were filtered at pH 4.8 according to the procedure used for riboflavin samples (13) until clear filtrates were obtained. When this was done, no "drift" (12) was noted nor was there any other evidence of disturbance of the assay results by stimulatory or inhibitory substances in the filtrates.

Materials to be digested were suspended in water, buffered to a pH appropriate to the enzyme being used and diluted to a final volume of 50 ml. In some cases the samples were given a preliminary autoclaving at pH 7.0. Unless otherwise noted, all samples were layered with toluene and incubated in 125 ml. Erlenmeyer flasks closed with cotton plugs. Those samples which were not to be treated with enzymes were prepared for assay by homogenizing in water, or by autoclaving in water suspension at pH 7.0 for 15 minutes at 121°C. In either case the mixtures were filtered as described above before assay.

The vitamin preparation used was synthetic *d*-calcium pantothenate. This was made up in a 50  $\gamma$  per ml. stock solution as directed for the microbiological method (7), and aliquots of the same solution were used both for the digestion experiments and to prepare the standard solutions for the necessary assays.

The biological materials studied were canned salmon, raw veal (round steak), canned lima beans, fresh milk, dried yeast and wheat bran. A sufficient quantity of each sample was set aside to last for the entire series of experiments. The wheat bran and yeast were preserved in the dry state, while the fresh samples were made into a slurry by homogenizing with water plus a small amount of chloroform, and were stored at  $-4^{\circ}\text{C}$ .

#### *a. Pantothenic Acid Content of Enzyme Preparations*

The enzymes used were clarase (Takamine Laboratories, Passaic, N. J.), mylase P (Wallerstein Laboratories, New York), polidase-S (Schwartz Laboratories, New York), papain (Merck), pancreatin (Merck) and a mixture of enzymes prepared by homogenizing mucosa from the upper end of the small intestine of a large adult rat with distilled water.

The pantothenic acid content of these enzyme preparations was determined after incubation under conditions similar to those used for the various samples. The results are given in Table I. The value

TABLE I

*Pantothenic Acid Content of Enzyme Preparations*

Enzyme Preparation	Conditions of Incubation			Pantothenic Acid Content $\gamma$ per g.
	Time Hr.	Temperature $^{\circ}$ C.	pH	
Clarase	24-72	37	4.8	5.2
Pancreatin	48	37	7.0	9.3, 9.6 <sup>a</sup>
Clarase and Pancreatin (1:1)	48	37	7.0	6.2
Papain	48	37	4.8	10.7, 16.5
Polidase S	48	37	4.8	1.4
Mylase P	24-72	37	4.8	2.0, 2.8
Mylase P	24	50	4.8	2.4
Rat Mucosa	48	37	<sup>b</sup>	16.2 <sup>c</sup>

<sup>a</sup> Individual values refer to different batches of enzyme.<sup>b</sup> pH not adjusted.<sup>c</sup> Based on fresh weight of mucosa. The other values are based on solid preparations as received.

for clarase is the average of 10 determinations on the same batch. Although the amount of the preparation in the mixtures incubated was varied from 0.1 to 1.0 g., and the time of incubation from 24 to 72 hours, all values fell between 4 and 6  $\gamma$  per g. except one which was 7. Other batches of clarase ranged in pantothenic acid content from 3.3 to 7.5  $\gamma$  per g.

#### *b. Effect of Enzymes on Calcium Pantothenate*

The percentages of the vitamin remaining after various enzyme treatments are shown in Table II. Each value is corrected for the pantothenic acid content of the enzyme as determined after incubation under similar conditions. The negative results mean that the correction in those cases was greater than the amount of pantothenic acid found in the digest. It is apparent that only clarase and mylase P permit fairly satisfactory recoveries under all the conditions tried. The amount of destruction in the other cases increased with increasing ratios of enzyme to vitamin. With polidase-S only the highest ratio tried caused destruction.

The two most promising preparations, clarase and mylase P, were then incubated with calcium pantothenate for varying lengths of time (Table III). Recoveries tended to be higher after the longer incubation

TABLE II

*Percentage Recoveries of Calcium Pantothenate from Enzyme Digests<sup>a</sup>*

Concentration of enzyme	Pantothenic Acid Added to Flask	Enzyme Preparation					
		Pancreatin	Papain	Polidase S	Mylase P	Clarase	Rat Mucosa <sup>b</sup>
<i>g. per 50 ml.</i>	$\gamma$	<i>per cent</i>					
0.01	5	87.2	81.6	102	97	115	
0.10	5	61.2	83	103	99.6	103	
1.00	5	negative <sup>c</sup>	negative	68	92	98.4	68
		negative	26				
1.00	25	66.8	73	88	89.3	116	88.8
		70.8					
1.00	250	80.5	97	98	95.6	90	95
1.00	500	100.3	120	117		90	

<sup>a</sup> 48 hrs. incubation at 37°C.<sup>b</sup> An amount equivalent to 108 mg. of mucosa was used in each flask.<sup>c</sup> In these cases the amount of residual pantothenate was less than that originally present in the enzyme used.

periods, and in several instances were well over the experimental error of the assay. Since it seemed unlikely that such increases could be attributed to the enzyme, a more detailed study of the 72 hour incu-

TABLE III

*Percentage Recoveries of Calcium Pantothenate after Enzyme Treatment for Various Times<sup>a</sup>*

Amount and Kind of Enzyme	Length of Incubation		
	24 hrs.	48 hrs. <i>per cent</i>	72 hrs.
Clarase, 1 g.	113	116	106
Clarase, 5 $\gamma$	110	113	124
Mylase P, 1 g.	117	137	141
Mylase P, 5 mg.	101	104	120
Mylase P, 5 $\gamma$	106	104	130

<sup>a</sup> Each flask contained 5  $\gamma$  of calcium pantothenate, and was incubated at 37°C.

bation period was made. Table IV summarizes the results. Satisfactory recovery of pantothenic acid activity was obtained in those mixtures which were not incubated and in those which were sterilized (by autoclaving or filtering) before incubation. However, there was a

slight but distinct tendency for the recoveries to be high after incubation of non-sterilized mixtures, either with or without enzyme present.

TABLE IV

*Recovery of Calcium Pantothenate after Incubation of Sterile and Non-Sterile Mixtures<sup>a</sup>*

Enzyme	Preliminary Treatment	Incubation Time	Pantothenic Acid Recovered
None	None	0	100, 104, 106, 106
None	15 min., 121°C.	0	105, 102, 103, 108
None	None	72	124, 119, 106, 94
None	15 min., 121°C.	72	106, 97, 84, 87
Clarase, 5 mg.	None	72	124, 123, 115, 87
Clarase, 5 mg.	Put through a bacterial filter	72	108, 100

<sup>a</sup> 5  $\gamma$  calcium pantothenate per flask; incubation temperature 37°C.

### c. Enzyme Treatment of Biological Materials

Table V shows the pantothenic acid content of the various biological materials studied, as determined after different preliminary treat-

TABLE V

*Pantothenic Acid Released from Biological Materials by Various Treatments<sup>a</sup>*

Treatment	Salmon	Veal	Lima Beans	Milk	Yeast	Wheat Bran
	$\gamma$ Pantothenic Acid per g.					
Suspended in water	7.3	12.1	0.9	2.4	54	31
Autoclaved at pH 7	8.3	10.1	1.1	2.7	48	32.4
Clarase "A" <sup>b</sup>	9.3	15.0	0.88	3.0	71.6	31.4
Clarase "B"	10.0	14.2	0.98	3.2	66.8	33.2
Clarase "C"	13.6	15.2	0.56	3.0	116 <sup>c</sup>	34.8 <sup>c</sup>
Pancreatin "A"	8.8	14.7	1.1	1.3	52.7	23.8
Pancreatin "B"	7.9	15.4	0.81	1.5	58.9	23.2
Pancreatin "C"	negative	negative	negative	negative	44.8 <sup>c</sup>	18.9 <sup>c</sup>
1:1 Mylase P—						
pancreatin "A"	8.0	10.1	1.0	1.5	38.4	29.5
"    "    "    "B"	7.7	14.7	1.2	1.5	45.6	39.9
"    "    "    "C"	negative	19.8	—	negative	47.4 <sup>c</sup>	31.9 <sup>c</sup>

<sup>a</sup> The enzyme digests were incubated 48 hours at 37°C.

<sup>b</sup> The amounts of enzyme used were based on the weights of sample taken, as follows: "A," one-tenth the dry weight; "B," equal to the dry weight; "C," twice the fresh weight.

<sup>c</sup> The amount of enzyme equaled twice the dry weight of sample.



ments. The quantities taken for analysis were: salmon and veal, 0.5 g. each; lima beans, 3.0 g.; milk, 1.0 g.; yeast and wheat bran, 0.1 g. each. The corresponding dry weights were estimated to be 0.17, 0.8, 0.13 and 0.1 g. respectively. The weights of the enzymes used were based on these figures as indicated in the table. Wherever necessary, the values are corrected as described above for the pantothenic acid content of the enzyme preparation used. Negative results mean that the value found was less than the correction. The digests containing clarase were buffered at pH 4.8 with sodium acetate, and those containing pancreatin were kept at pH 7 with a phosphate buffer. Where both mylase P and pancreatin were used, the samples were first incubated 24 hours at pH 7 with pancreatin, then heated to 100°C. for 45 minutes, cooled, adjusted to pH 4.8 and incubated with mylase P an additional 24 hours.

It is apparent that destruction of the vitamin occurred in many of the digests containing pancreatin. Of those containing clarase, salmon and yeast showed increases up to the maximum amount of enzyme tried, while the rest showed little variation. No explanation for the low value of 0.56 for lima beans can be offered.

Table VI shows the liberation of pantothenic acid by clarase and mylase P when the samples were incubated with a given amount of

TABLE VI

*Pantothenic Acid Liberated by Clarase and Mylase P after Various Periods of Incubation*

Sample	Mylase <sup>a</sup> P 50°C. incubation				Clarase <sup>a</sup> 37°C incubation			
	1 hour	8 hours	12 hours	24 hours	2 <sup>b</sup> hours	24 hours	45 hours	72 hours
	<i>γ of pantothenic acid per g.</i>							
Salmon	8.5	7.1	10.8	11.3	6.9	9.6	9.1	10.1
Veal	11.7	12.1	12.3	16.9	11.5	13.2	14.2	13
Milk	2.7	2.7	3.0	3.4	3.1	2.5	3.3	3.4
Lima beans	1.0	1.0	1.1	1.2	0.8	1.1	1.1	1.7
Yeast	65	79	93	124	53	104	112	
Wheat bran	35	34	36	40			33	

<sup>a</sup> The amounts of enzyme were: Mylase P as in "A," Table V; Clarase as in "B," Table V. The Mylase P results refer to aliquots taken from the same digests, while the clarase results apply to separate digests.

<sup>b</sup> 50°C. incubation.

enzyme for varying lengths of time. All of these digests were adjusted to pH 4.8 with sodium acetate before incubation. In general it appears that similar maximum values are reached with much less mylase P than clarase and in a shorter time. Incubation with clarase at 50°C. for more than 2 hours was not tried. The flasks to be incubated at 50°C. were corked rather than plugged with cotton to prevent loss of toluene.

### RECOMMENDED PROCEDURE

An amount of the finely divided sample estimated to contain between 3 and 10  $\gamma$  of pantothenic acid is suspended in 50 ml. of water, the suspension is adjusted to pH 6.8-7.0, and autoclaved for 15 minutes at 121°C. After the mixture has reached room temperature an amount of mylase P equivalent to one-tenth the estimated dry weight of the sample is added to the flask. The contents are buffered with 2 ml. of 2.5 *M* sodium acetate, and the pH lowered to 4.8 with hydrochloric acid. The mixture is then layered with toluene, corked and incubated for 12-24 hours at 50°C. At the end of the incubation period, the volume of the digest is increased to 100 ml. with distilled water, and it is put through a fluted Whatman No. 40 paper, repeatedly if necessary, until a clear filtrate is obtained. The pH is checked before filtration and again adjusted to 4.8 if necessary. After a clear filtrate has been obtained, it is further diluted to a convenient concentration for assay.

### DISCUSSION

The recommended procedure detailed above represents the best suggestions that can be made on the basis of the present work. Mylase P has been selected since it appears to act more quickly and in smaller amounts than the others tried, and because its pantothenic acid content is so low that the blank correction is negligible. It is felt that the two hours incubation period which has been suggested for mylase P (2) is inadequate for maximum results. However, incubation for as long as 72 hours seems equally undesirable because of the danger of high and erratic results in the non-sterile mixtures incubated.

### SUMMARY

Digestion with mylase P for 12-24 hours at 50°C. is recommended for samples to be assayed for pantothenic acid. Several other enzymes are shown to destroy pantothenic acid if used in relatively large amounts.

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# The Effect of Various Reagents on Adrenocorticotrophic Hormone \*

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## INTRODUCTION

Recently the anterior hypophyseal adrenocorticotrophic hormone from both sheep (1) and swine (2) pituitaries has been isolated in highly purified state. In contrast to other protein hormones, the adrenocorticotrophic hormone retains its biological properties after its neutral solution has been kept at 100°C. for 2 hours; it was further noted that peptic digestion does not destroy the specific action of the hormone (1). It was felt that the study of the effect of various reagents upon the purified hormone would provide additional information as to its structural characteristics.

### *Action of Ketene*

Adrenocorticotrophic hormone used in the present investigation was prepared by the procedure of Li *et al.* (1). The hormonal activity was estimated either by the maintenance of adrenal weights of hypophysectomized male rats or by the repair of adrenal histology in hypophysectomized female rats as previously described (3). Ketene was generated by the apparatus of Li (4); experiments were carried out at 0°C. with the protein concentration of 10 mg. per cc. in two buffer solutions: 1.0 *M* acetate buffer of pH 4.0 and 0.75 *M* phosphate buffer of pH 7.0. The acetylated hormone solutions were dialyzed against distilled water and then lyophilized.

The groups in proteins known to react with ketene are the free

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amino, the tyrosine phenolic and the —SH groups (5). The amino nitrogen content of adrenocorticotrophic hormone as determined by the manometric method of Van Slyke was found to be 0.70%. It contains no —SH groups (1). Hence, the reaction of ketene with the hormone must be confined to the free amino and tyrosine phenolic groups in the molecule.

After 5 minutes treatment with ketene in *phosphate buffer* it was found that one fifth of the phenolic group in adrenocorticotrophic hormone was acetylated as determined by the method of Herriott<sup>1</sup> (6) while the loss of amino nitrogen was, at maximum, 50%. When the treatment was prolonged to 50 minutes, sixty % of the amino groups and thirty-five % of the phenolic hydroxyl groups were acetylated. The results of bioassay, as summarized in Table I, indicate that the biological potency of the hormone was lowered as the time of acetylation increased. It may be noted that there was no great difference in the extent of coverage of the phenolic group after 2 to 5 minutes treatment yet the adrenocorticotrophic activity was continuously decreased. It appears reasonable, therefore, to conclude that the continuous loss of hormonal action should be at least partly attributed to acetylation of the free amino groups.

When the hormone reacted with ketene in *acetate buffer* for 15 minutes, the extent of coverage of the amino groups was 35%.<sup>2</sup> With longer treatment (160 minutes), no further acetylation of the amino group was observed, whereas the extent of acetylation of the phenolic hydroxyl group increased from 30 to 50%. From the results shown in Table I, the preparation after 15 minutes treatment gave a minimum effective response at 1.0 mg. while after 160 minutes treatment no adrenocorticotrophic activity could be detected at a total dose of 1.5 mg. It may, therefore, be concluded from the experiments with ketene

<sup>1</sup> The method of Herriott is based on the fact that oxygen-acetyl combination in the tyrosine is easily hydrolyzed at pH 11; the ratio of the Folin color obtained by the pH 8 method to that by the pH 11 method indicates the extent of acetylation of phenolic hydroxyls in the preparations. It may be mentioned that complete Folin color recovery was obtained in the acetylated hormone samples after pH 11 treatment.

<sup>2</sup> It may be noted in Table I that the rate of acetylation of both amino and tyrosine phenol groups is higher at pH 7.0 than at pH 4.0 and that the amino group reacts with ketene in acetate buffer seemingly more slowly than the phenol hydroxyl groups. The latter fact appears to contradict current views that amino groups are always more rapidly ketenized than the tyrosine hydroxyls; more data are needed to substantiate this conclusion.

TABLE I

*The Action of Ketene on Adrenocorticotrophic Hormone*

Experiment No.	Solvent	Time of Treatment	pH		Free NH <sub>2</sub> Groups Covered	Phenol Groups Covered	Assay in Hypophysectomized Female Rats*	
			Start	Final			Total Dose	Adrenal Reaction
93C	Phosphate buffer	min. 2	7.0	6.7	per cent 10	per cent 20	mg. 0.40	2+, 2+, 2+
							0.20	+, +, +
							0.10	-, +, +, -, -, +
							0.05	-, -, -
85B	Phosphate buffer	3	7.0	6.6	30	20	1.0	2+, 2+, 2+
							0.5	2+, 2+, 2+
							0.2	+, +, +
							0.1	-, -, -
87A	Phosphate buffer	5	7.0	6.5	50	20	1.5	+, +, +
							1.0	+, -, -, -
							0.2	-, -, -
87B	Phosphate buffer	50	7.0	4.2	60	35	2.0	-, -, -
							1.0	-, -, -
03A	Acetate buffer	15	4.0	3.8	35	30	1.0	+, +, +
							0.5	-, -, -
03B	Acetate buffer	100	4.0	3.5	35	50	1.5	-, -, -
							0.5	-, -, -
09	Acetate buffer	0	4.0	4.0	0	0	0.10	2+, 2+, +
							0.05	+, +, +

\* Female rats are hypophysectomized at 26 to 28 days of age; 14 days later the rats are injected intraperitoneally once daily for 4 days, followed by autopsy 96 hours after the first injection.

that both the phenolic hydroxyl and amino groups are essential for the characteristic effect of the adrenocorticotrophic hormone.

#### *Action of Nitrous Acid*

Forty mg. of adrenocorticotrophic hormone were dissolved in 2 cc. of 1.0 M acetate buffer of pH 4.0 and 0.5 cc. of 4 M NaNO<sub>2</sub> solution

was added. The solution was allowed to stand at 22°C.; at the end of 30 minutes, the solution was neutralized and then assayed in hypophysectomized male rats. As shown in Table II (Expt. 01), adrenocorticotrophic activity was completely abolished.

In another experiment (Expt. 61 in Table II) 80 mg. of hormone was dissolved in 2 cc. acetate buffer with 0.5 cc. nitrite solution and further handled as above. Assay results indicate again that the biological potency of the hormone is greatly reduced by this treatment.

TABLE II  
*The Action of Nitrous Acid on Adrenocorticotrophic Hormone*

Experiment No.	Amount of Hormone in 2 cc. Acetate Buffer	Vol. of 4.0 M NaNO <sub>2</sub>	Time of Reaction	Assay in Hypophysectomized Male Rats <sup>1</sup>			
				Daily Dose	No. of Rats	Average Body Weight	Average Adrenal Weight
	mg.	cc.	min.	mg		g.	mg.
01	40	0.5	30	0.5	5	123	12
61	80	0.5	30	1.0	4	120	20
09	40	0.0		0.2	10	128	26

<sup>1</sup> Male rats, 40 days of age, are hypophysectomized and injected intraperitoneally daily (except Sunday) from the day of operation for 15 days (13 injections). The adrenal weight of uninjected hypophysectomized animals regresses during this period from about 26 mg. to a weight of 12 mg.

Nitrous acid is known to react with free amino and tyrosine groups in proteins in addition to its oxidizing action. Philpot and Small (7) have found that the amino nitrogen of pepsin was completely liberated within 30 minutes by nitrous acid treatment and that the secondary reaction with tyrosine was scarcely started by this time. If we assume that the behavior of adrenocorticotrophic hormone toward nitrous acid resembles that of pepsin, further evidence is furnished that the free amino group in adrenocorticotrophic hormone is necessary for its biological activity.

#### *Action of Formaldehyde*

It is well known that formaldehyde reacts mainly with the free amino group of proteins in neutral solutions. In order to sustain the findings with ketene and nitrous acid, it seemed desirable to treat

adrenocorticotropic hormone with formaldehyde. Sixty mg. of the hormone was dissolved in 10 cc. of 1.0 *M* phosphate buffer of pH 7.0 and 1 cc. of 50% formaldehyde solution was added. The solution stood at 22°C. for 30 minutes and was then diluted for bioassay in hypophysectomized male rats. With a daily dose of 0.50 mg. of treated hormone for 15 days, the adrenal weights were not increased over those of untreated controls; they averaged only 14 mg. (5 rats) while with a daily dose of 0.20 mg. of the untreated hormone, the adrenal weights (5 rats) were 24 mg. It thus again appears that free amino groups are important for the biological action of adrenocorticotropic hormone.

### *Action of Iodine*

If a protein contains no —SH groups, it takes up iodine through the tyrosine group (8). Since, as previously mentioned, adrenocortico-

TABLE III  
*The Action of Iodine on Adrenocorticotropic Hormone*

Experiment No.	Vol. of 0.01 <i>N</i> Iodine Added to the Hormone Solution* cc.	Assay in Daily Dose mg.	Vol. of 0.01 <i>N</i>	Male Rats**
			Average Body Weight g.	Average Adrenal Weight mg.
15	5.0	0.5	122	12
11	4.6	0.5	120	15
4A	3.5	0.2	124	14
4B	1.7	0.2	120	19
4C	0.0	0.2	121	22
4D	0.0	0.5	122	28

\* 40 mg. adrenocorticotropic hormone dissolved in 15 cc. pH 7.0 phosphate buffer.

\*\* 5 animals per group in each experiment.

tropic hormone does not possess cysteine and since it contains approximately 4.5% of tyrosine as determined by the method of Lugg (9), it seemed of interest to investigate the effect of iodine on the physiological activity of adrenocorticotropic hormone.

Forty mg. of adrenocorticotropic hormone were dissolved in 15 cc. of 0.3 *M* phosphate buffer of pH 7.0 and 5.0 cc. of 0.010 *N* iodine solution<sup>3</sup> were added. After one hour at 22°C., the slight excess of

<sup>3</sup> If all tyrosine groups in 10 mg. of adrenocorticotropic hormone are completely iodinated, 1 cc. of 0.010 *N* iodine solution is needed.



iodine was removed by a drop of 0.10 *N* sulfite solution. The iodinated hormone solution was diluted and then assayed in hypophysectomized male rats. As shown in Table III, the adrenocorticotropic activity completely vanished.

In other experiments we added decreasing amounts of iodine to the hormone solution. It was found that the biological potency of the hormone decreased as the amount of iodine uptake increased, as indicated in Table III. The loss of adrenocorticotropic activity seems to be due to the iodination of the tyrosine group in the hormone.

### SUMMARY

The effect of ketene, nitrous acid, formaldehyde and iodine on the adrenocorticotropic hormone has been studied. The results suggest that both the free amino and tyrosine groups are essential for the hormonal action.

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# Coccarboxylase as a Growth Factor for Certain Strains of *Neisseria gonorrhoeae*

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## INTRODUCTION

Certain freshly-isolated strains of the gonococcus may manifest important variations from the "normal" in their nutritive requirements. Any diagnostic culture medium which aims for high productivity must, therefore, supply sufficient quantity of such additional growth factors as may be required by these "atypical" strains. Thus, glutamine is a necessary supplement to Bacto Proteose Peptone No. 3—hemoglobin agar for primary isolation of about 25% of the cultures obtained from patients in this area (1, 2). Certain other strains will grow upon this medium only in the presence of a relatively thermostable factor present in yeast and other natural substances. It was observed that, although this factor may be replaced by a relatively high concentration of thiamine, the high activity of yeast extract could not be explained on the basis of its thiamine content alone (3). The evidence now available indicates this thermostable factor of yeast extract to be cocarboxylase.

## METHODS

The cultures employed in this study were isolated from cervical specimens plated upon Bacto Proteose Peptone No. 3—hemoglobin agar containing adequate nutritive supplements. Positive cultures were purified and tested for growth upon two plates of the base medium supplemented with glutamine and yeast extract, respectively. An occasional culture was found to grow on the plate containing autoclaved yeast extract, but failed to grow on the glutamine-containing medium. Altogether, 36 such cultures have been identified, of which 17 were used in various phases of this study. These cultures were identified as *Neisseria gonorrhoeae* by their typical morphology, oxidase activity and colony characteristics in the presence of an optimum

quantity of yeast extract; their alkali solubility; their capacity to ferment only glucose in a suitable medium; and their production of variants similar in every respect to the "normal" type of gonococcus.

Throughout most of this investigation, the Bacto Proteose Peptone No. 3—hemoglobin agar was used as a test medium, since the cultures in question produced no trace of growth from light inocula unless suitable supplements were included. For the quantitative studies, 5 mg. % of cystine was added in some instances to increase the range of colony size (3).

Pure chemical compounds to be tested for growth-promoting activity were dissolved in sterile water and diluted as required without subsequent sterilization. Contamination was negligible. The yeast extracts were sterilized by Seitz filtration, however. Appropriate dilutions of the test substance were pipetted to sterile plates and mixed thoroughly with a measured quantity of agar (usually 25 ml.).

Estimation of growth response was more or less subjective in the early phase of the study. Sectors of each plate in a series of concentrations of the test substance were streaked with a standard loopful of broth suspension of the exacting cultures. A "normal" strain was always included for the dual purposes of registering any toxicity of the test substance and of serving as a standard of "optimum" growth. The amount of growth after incubation at 35°C., usually for 24 hours, was estimated and recorded as indicated in Table I. When comparisons were to be made of the activity of two different substances, the plates of each test series were compared with each other. The results were fairly reproducible in separate experiments if the successive decreases in concentration were not less than 0.5-fold.

A method for estimating average colony diameter of the gonococcus as a function of growth response, as suggested by Gould (4), has been adapted to this study, with certain modifications. A heavy suspension of an 18 hour culture was prepared in 2 ml. of starch broth, and serial dilutions were made in the same medium. From the 1:1,000,000 dilution, 0.1 ml. was pipetted to each plate of the test series and spread evenly with an L-shaped glass rod, which was sterilized by immersing in alcohol, then burning away the adhering alcohol. The excess surface moisture on the inoculated plates was dried by inverting the plates in the incubator for 15 to 30 minutes with tops removed. Following 48 hours incubation, the diameter of 20 colonies on each plate was measured with a low-power binocular microscope fitted with an ocular micrometer. A bright light was necessary to illuminate the translucent edges of the colonies, and the oxidase reagents were helpful in visualizing the smaller colonies. In few of the experiments was the number of colonies less than 30 or more than 120. In any one series the variation in number rarely exceeded  $\pm 20\%$ . This method of plating allows a uniformity of colony distribution not possible with the streaking method used by Gould; moreover, elongation of the colonies along the line of streak is eliminated (Fig. 1).

The proportionality of colony diameter to concentration of growth factor may be influenced by a number of conditions. For example, crowding greatly reduces colony size, so that only well separated colonies should be measured. Colonies near the edge of the plate may be affected by the capillary rise of the agar on the side wall. Uniformity in depth of agar may be controlled by using flat-bottom plates and 25 ml. of agar. It is very important to stabilize the test strain to the colonial type which

forms large, circular, entire colonies. Another factor, over which we have not been able to exercise entirely satisfactory control, is the degree of drying of the agar surface. If the agar dries until lines of strain begin to show, the colonies will not develop to proportionate size. Close supervision of the drying process is necessary to avoid significant error from this source.

TABLE I  
*Estimation of Growth Response to Thiamine and Yeast Extract*

Supplement	Concentration	Strains			
		3071	1131	831	2740 (N)
0	--	—	—	—	++++
Thiamine (B <sub>1</sub> A)	mg per cent				
	5.0	++++	++++	++++	++++
	1.0	++	++	++	++++
	.4	+	+	+	++++
	.1	±	±	+	++++
	.04	±	±	±	++++
	.01	—	—	—	++++
Yeast extract*	per cent				
	.2	++++++**	++++++	++++++	++++++
	.04	++++	++++	++++	++++
	.004	++	++	++	++++
	.001	±	±	+	++++
	.0002	±	±	±	++++
	.0001	—	—	±	++++

\* Per cent equivalent of dried yeast.

\*\* Non-specific stimulation.

(N) "normal" strain.

The source of the samples of thiamine chloride hydrochloride used in this study is indicated in Table II. Thiamine disulfide was prepared according to the method of Zima and Williams (5) and melted at 170°C., uncorrected. The 2-methyl-5-sulfonylmethyl-6-aminopyrimidine ("pyrimidine") and 4-methyl-5-β-hydroxyethyl-thiazole hydrochloride ("thiazole") were prepared from thiamine by sulfite cleavage and subsequent crystallization (6). Thiamine monophosphate was obtained by heating a *N* HCl solution of cocarboxylase (Merck, synthetic) in a water bath at 100°C. for 30 minutes, after the method of Lohmann and Schuster (7).

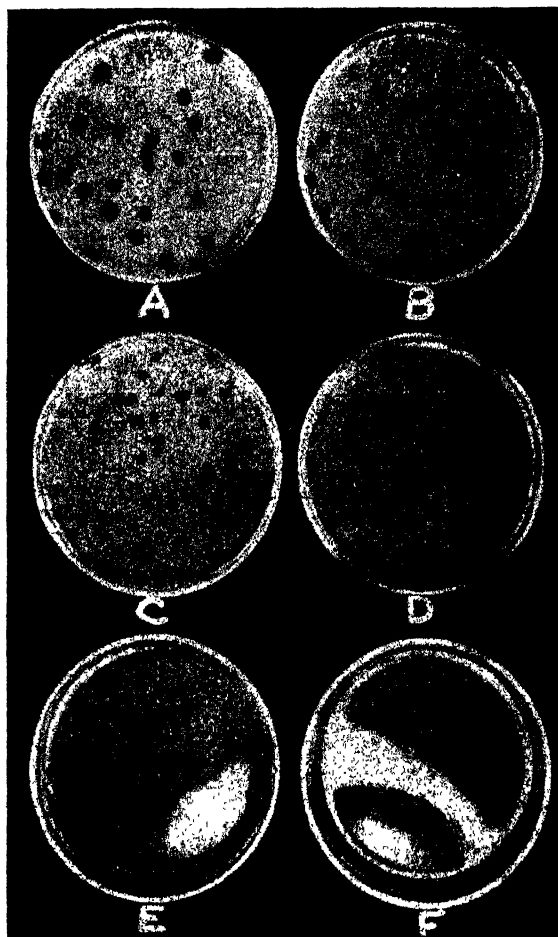


FIG. 1

#### Colony Diameter as a Function of Cocarboxylase Concentration

The medium is Proteose Peptone No. 3—hemoglobin agar plus 5 mg. % cystine. Plates were inoculated with a 0.1 ml. of 1:10,000,000 dilution of culture suspension. All plates were "developed" with the oxidase reagent, dimethyl-*p*-phenylenediamine hydrochloride. The concentration of cocarboxylase in each plate is: A, 0.25%; B, 0.15%; C, 0.08%; D, 0.01%; E, 0.005%; and F, no cocarboxylase.

## EXPERIMENTAL RESULTS

*Properties of the Thermostable Yeast Factor* The type of gonococcus considered in this study was first observed during a survey of the incidence of those strains which require a thermolabile factor of yeast extract (glutamine) for growth. It was noted that certain strains, for which the yeast extract supplement to the base medium was a requisite



FIG 2

## Satellite Stimulation of Cocarboxylase-Deficient Gonococci

The agar base contains 0.008% cocarboxylase. The two colonies of staphylococcal contaminants (white colonies) show marked zones of stimulation for the gonococcus (black colonies).

for growth, would develop even though the extract had been autoclaved, a treatment which is destructive to glutamine. These strains would grow also as satellites to a number of contaminating bacteria (Fig 2); or if the diagnostic specimen contained considerable pus or mucus, very small colonies might be observed on some areas of the unsupplemented base medium. It was found that a number of natural

substances would supply the deficient nutritive: liver extract, beef muscle extract, human and rabbit blood, human urine and cow's milk. Two lots of Bacto desiccated yeast extract were inactive, however.

At this point an attempt was made to substitute known growth factors, singly and in combination. The substances tested were: thiamine hydrochloride, nicotinic acid, nicotinamide, cozymase I, riboflavin, calcium pantothenate, *p*-aminobenzoic acid, pyridoxine hydrochloride and a folic acid preparation (10  $\gamma$  %, each): choline hydrochloride, adenine sulfate, guanine hydrochloride, xanthine, hypoxanthine, uracil, "thymine," cytosine and adenine thiomethylpentose (100  $\gamma$  % each); inositol, glutathione and cystine (1 mg. % each); and biotin (0.01  $\gamma$  %). None of these substances exhibited any activity, except the "thymine" preparation, which later proved to be mislabeled *thiamine*. Before this error in identity was discovered, however, synthetic thymine, thymosine, 5-methylcytosine, and yeast- and thymonucleic acids were tested, also without effect. The failure of the synthetic thymine to substitute led to further study of the yeast extract.

The yeast factor was not precipitated by mercuric acetate at an acid reaction, nor was it appreciably absorbed by Norit at pH 4. Its activity was only slightly diminished following autoclaving in *N* H<sub>2</sub>SO<sub>4</sub>, but was completely destroyed by *N* NaOH under the same conditions. Incubation of the extract with sodium sulfite at pH 5 destroyed approximately 99% of its activity, and treatment with alkaline ferricyanide had a similar destructive effect.

*The Activity of Thiamine and its Derivatives.* With this suggestive information at hand, thiamine hydrochloride was retested in higher concentration, and the active "thymine" was identified as thiamine (9). In concentrations of 5 mg. %, or better, the thiamine hydrochloride could completely replace the yeast extract (Table I), and its stimulatory effect could be detected in concentrations as low as 0.05 mg. %. The necessity for such an unusually high concentration of a growth factor to support optimum growth suggests that it is not the only active compound supplied by such natural substances as yeast and tissue extracts. In fact, the activity of yeast extract cannot be explained on the basis of its thiamine content alone, since the dry weight of a concentrated extract required to produce the maximum effect (ca. 1.2 mg. %) is less than the quantity of thiamine (5 mg. %) necessary for equivalent growth stimulation. It seemed a reasonable

assumption, therefore, that cocarboxylase might be the active compound present in the yeast and tissue extracts. Before this supposition could be tested, however, certain other interesting observations were made.

During a later experiment, in which a different sample of thiamine was being used, no growth promoting effect on the same gonococcal strains could be detected. Other samples of thiamine were tested subsequently, with results indicated in Table II. Two samples, labeled

TABLE II  
*Comparison of Activity of Thiamine Samples*

Sample	Concentration for maximum effect	Concentration for minimum effect	Per cent activity of A	Source	Date of preparation
	<i>mg per cent</i>	<i>mg per cent</i>			
A	5.0	.05	100	SMACO (Merck)	May, 1941
B	50	.5	ca. 10	SMACO (Merck)	Dec., 1942
C	—	—	0	Merck	Spring, '43
D	—	—	0	Eastman	?
E	50	.5	ca. 10	SMACO (Merck)	1942
F	10	.1	ca. 50	Eastman	?
N*	1.0	.01	ca. 500	Merck	"old"
G**	10	.1	ca. 50	J. K. Cline	"old"

\* Natural thiamine.

P<sub>1</sub>\* Thiamine bromide hydrobromide.  
th

P<sub>1</sub>C and B<sub>1</sub>D, were inactive in the highest concentration tested (100 mg. %). Samples B<sub>1</sub>B and B<sub>1</sub>E possessed approximately 10% of the activity of the original reference sample, B<sub>1</sub>A; while B<sub>1</sub>F and B<sub>1</sub>G were about 50% as effective as the reference compound. On the other hand, an "old" sample of natural thiamine (Merck) possessed about 5-fold the activity of B<sub>1</sub>A and produced somewhat larger colonies (Fig. 3).

The cause of this marked difference in activity of the eight thiamine samples has not been determined. Several possibilities are suggested, however: (1) that the active samples may be contaminated with small quantities of a highly active compound (e.g., cocarboxylase) either



during its preparation or as the result of mold or bacterial growth during storage; (2) thiamine may undergo some chemical transformation such as oxidation or cleavage, with resultant formation of an active compound; (3) exposure to light or to laboratory gases may be responsible for formation of active derivatives; and (4) the inactive samples may contain an inhibitor which antagonizes any inherent activity of the thiamine. This last possibility was eliminated by mixing varying proportions of B<sub>1</sub>A and B<sub>1</sub>C in the test medium; in every instance the growth response was practically equivalent to that

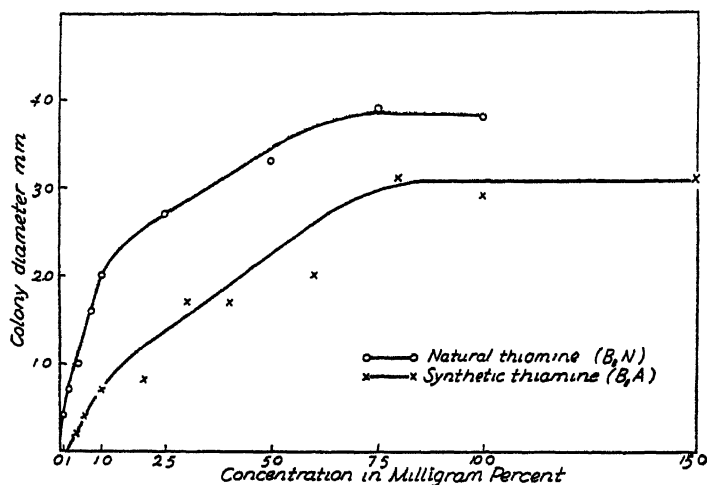


FIG. 3

Growth Response of Cocarboxylase-Deficient Strains to Certain Samples of Thiamine Hydrochloride

obtained with B<sub>1</sub>A alone. The third possibility has not been tested, although it is certainly worthy of consideration. No evidence of significant contamination by molds or bacteria was obtained; in fact, unfiltered solutions of all samples were incorporated into the agar base with negligible contamination. Moreover, microscopic examination of concentrated solutions failed to reveal any fragments of fungal hyphae. In consideration of the improbable chance that synthetic thiamine might be contaminated with mono- or diphosphothiamine during its preparation, an attempt was made to eliminate this remote possibility. This will be discussed later.

The activity of several oxidation and cleavage products of thiamine has been investigated (Table III). Neither thiochrome (Merck) nor thiamine disulfide showed more than a trace of activity. The "pyrimidine" fraction of thiamine was completely inactive, while "thiazole" was found to be 1 to 2% as effective as B<sub>1</sub>A. An equimolecular mixture of the two cleavage products was no more active than the thiazole moiety alone. Unlike the thiamine samples, however, the thiazole did not promote growth of all test strains to an equal degree. In this connection it is interesting to note that sulfite cleavage of an ineffective thiamine preparation (B<sub>1</sub>C) resulted in acquisition of slight activity, equal to that possessed by the thiazole product, whereas similar treatment of B<sub>1</sub>A destroyed all activity except the 1 to 2%

TABLE III  
*Activity of Thiamine Derivatives*

Derivative	Per cent activity of reference thiamine sample (B <sub>1</sub> A)
"Pyrimidine"	0
"Thiazole"*	ca. 1.0
Pyrimidine + thiazole	ca. 1.0
Thiochrome	< 1.0
Thiamine disulfide	< 1.0

\* Some strains show greater response than others.

attributable to the thiazole. When autoclaved in solution at pH 2.3 the activity of B<sub>1</sub>A was diminished but slightly, while autoclaving at pH 9.6 destroyed all growth-promoting effect. It seems evident from these results that none of the more familiar oxidation and cleavage products of thiamine are responsible for the activity of the effective samples.

These observations do not eliminate the possibility that thiamine may undergo some chemical transformation during aging or exposure to a variety of conditions in the laboratory. Indeed, a check on the age of the several samples does suggest such a contingency. In Table II it may be noted that the activity is directly related to the age of at least some of the specimens. The sample of natural thiamine is probably older than any of the synthetic compounds. Moreover, when B<sub>1</sub>B was first tested in December 1943, it was devoid of effect, yet when retested in August 1944 with the other samples, it possessed an activity of

about 10%. Two of the same test strains of gonococcus were used on both occasions.

*Coccarboxylase and Thiamine Monophosphate.* When a sample of coccarboxylase (Merck, synthetic) was tested, it became evident at once that the high activity of the several tissue extracts could be explained on the basis of their content of this coenzyme. A concentration of 0.1  $\gamma$  % coccarboxylase supported optimum growth of all 12 test strains, as determined by the streak plate method, and a trace of

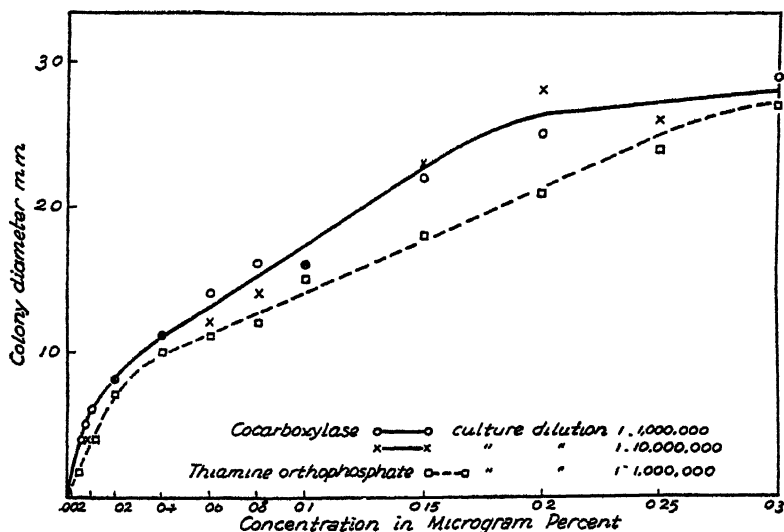


FIG. 4

Growth Response of Coccarboxylase-Deficient Strain to Coccarboxylase and Thiamine Orthophosphate

growth was detected with as little as 0.001  $\gamma$  %. From this point in the study, the average colony diameter was used as a quantitative expression of growth response. By this means the growth response of strain No. 1436 to coccarboxylase and to thiamine monophosphate was compared (Fig. 4). Quantities of thiamine monophosphate are given in terms of coccarboxylase in order that comparisons on a molar basis may be made. From the results of several tests it was found that the activity of the monophosphate is about 80% that of coccarboxylase, although this value varies somewhat with the portion of the curves

used for comparison. In this and subsequent determinations the maximum colony diameter was obtained with 0.2  $\gamma$  % cocarboxylase, and very minute colonies could be observed on the plate containing 0.001  $\gamma$  %.

*The Effect of Phosphatase (Takadiastase) upon Cocarboxylase and Tissue Extracts.* If the growth promoting effect of various tissue extracts is due to their content of cocarboxylase (or thiamine monophosphate), their activity should be eliminated largely by phosphatase cleavage. To test this effect, cocarboxylase and several tissue extracts were incubated with takadiastase for 24 hours after the method described by Cheldelin and associates (8), except that a fluid extract of takadiastase (44 mg. per ml.) was used. The results, as presented in Table IV, demonstrate that all the natural substances lose the major portion of their activity when treated with takadiastase, although the residual activity varied widely with different substances. While cocarboxylase retained only 0.1% of its original potency following takadiastase treatment, the tissue extracts retained 1 to 2% of their activity, and yeast extract and red blood cells lost only 80 to 90% of their original equivalent of cocarboxylase. Since the dephosphorylation of cocarboxylase by phosphatase is an equilibrium reaction, the differences observed in residual activity of the various extracts may be due, in part at least, to their relative concentrations of free thiamine and cocarboxylase before the phosphatase is applied. It is not certain, however, that the relatively great residual activity of the yeast extract and blood cells can be explained entirely on this basis, although considerable free thiamine must be present in an extract of dried yeast owing to the action of yeast phosphatase. Other substances possessing growth-promoting effect might be present, however; or cocarboxylase may be present as a firmly bound form not readily attacked by phosphatase (10).

The extent of inhibition of dephosphorylation of cocarboxylase by free thiamine is shown in Fig. 5. The results were obtained by mixing thiamine and cocarboxylase (the quantity of the latter remaining constant) in the ratios indicated, incubating 24 hours with takadiastase, and assaying the residual cocarboxylase with strain No. 1436. For ratios of thiamine to cocarboxylase below 100, the inhibitory effect on phosphatase cleavage appeared to be slight and fairly constant, but for ratios above this level the residual cocarboxylase rises significantly. Westenbrink and van Dorp (12) observed marked inhibition

of phosphatase with a much smaller quantity of thiamine and pyrimidine, but their assays were conducted within 30 minutes after the addition of the phosphatase, and their time curves of cocarboxylase

TABLE IV  
*Cocarboxylase Assay Values of Natural Materials Before and  
After Takadiastase Treatment*

Material	Concentration, g /100 ml * of agar	Colony diameter, mm	Equivalent of cocarboxylase ( $\gamma$ )	Average, $\gamma$ /g.	Per cent destroyed by takadiastase
Beef Muscle	.4	1.8	.21	.22	99
	.2	1.2	.22		
	.1	.9	.22		
Pork Muscle	.1	2.5	1.32	1.27	99
	.04	1.3	1.25		
	.02	1.0	1.25		
Beef Liver	.2	2.3	.6	.73	98.4
	.1	1.8	.84		
	.04	1.0	.75		
Urine No. 1	10.0%	.8	.18	.15 $\gamma$ per 100 ml.	—
	7.5	.6	.13		
Urine No. 2	10.0%	.6	.1	.1 $\gamma$ per 100 ml.	—
	7.5				
Red Blood Cells (Human)	.5	1.2	8.6	9.9 $\gamma$ per 100 ml. blood	80
	.25	1.0	11.2		
	.1	.6	10.0		
Plasma** (Human)	10%	0	0	0	—

\* Wet weight.

\*\* No growth at highest test level.

hydrolysis, with and without thiamine or pyrimidine, tend to converge within this short period.

If the growth promoting effect of the several active samples of thiamine were due to contamination by traces of cocarboxylase or thiamine monophosphate, this activity should be destroyed by taka-

diastase, within the limit imposed by the relatively high ratio of thiamine present. For sample B<sub>1</sub>A it was calculated that 1 part cocarboxylase to 40,000 parts thiamine would have to be present to account for the activity of this sample. Referring to Fig. 5, it was estimated that takadiastase should destroy 95% of the activity in 24 hours. Repeated tests with this sample, however, failed to show any detectable diminution of activity during 24 hours exposure to taka-diastase. Hence, it seems unlikely that phosphothiamine can be responsible for the activity of this sample.

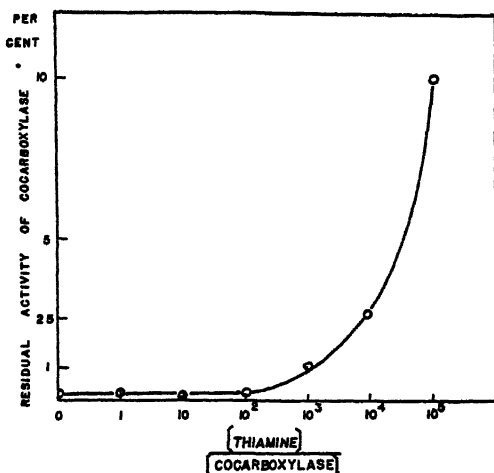


FIG. 5

Inhibition of Phosphatase Cleavage of Cocarboxylase by Thiamine

*The Inhibition of Cocarboxylase Utilization by Thiamine.* A number of compounds are known to inhibit the synthesis and functioning of the carboxylase system. Buchman, *et al.* (13), for example, demonstrated that thiazole diphosphate markedly inhibits the decarboxylation of pyruvate by alkaline-washed yeast in the presence of added cocarboxylase. This inhibition was explained on the basis of a "competition between cocarboxylase and thiazole pyrophosphate for the specific carboxylase protein with which the two are similarly able to combine." According to Weil-Malherbe (14), adenosine triphosphate in excess similarly inhibits cocarboxylase as a result of competition for the apoenzyme. More recently, Woolley and White (15) and Wyss

(16) have demonstrated that pyrithiamine, the pyridine analogue of thiamine, competitively inhibits the utilization of thiamine, particularly by microorganisms for which the latter is an essential growth factor. Sarett and Cheldelin (17) have reported that pyrithiamine and 6-aminopyrimidine compounds inhibit the utilization of cocarboxylase by *Lactobacillus fermenti* more than they inhibit the use of thiamine. It might be expected that pyrithiamine and 6-aminopyrimidine would antagonize phosphothiamine utilization by the gonococci considered here. Moreover, it is also quite possible that thiamine itself might exert some antagonism, since it appears to be metabolically inert for these organisms, yet by mass action it might saturate the apoenzyme

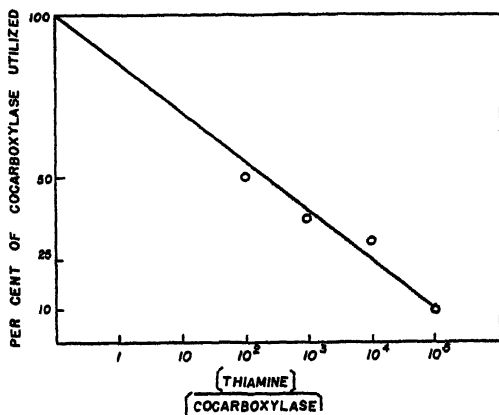


FIG. 6

Inhibitory Effect of Thiamine on Cocarboxylase Utilization

in competition with the functionally active thiamine diphosphate. This possibility was tested by mixing thiamine and cocarboxylase in the proportions indicated (Fig. 6), then assaying for the effective equivalent of cocarboxylase. When the ratio of thiamine to cocarboxylase was  $10^5$ , about 10 times as much cocarboxylase was required to produce growth equivalent to that obtained in the absence of thiamine. Smaller quantities of thiamine necessitated proportionately smaller additions of cocarboxylase to effect the same growth response.

*Production of "Normal" Variants of the Cocarboxylase-Deficient Strains.* Unless the cocarboxylase-deficient strains are cultivated on a medium containing the optimum quantity of this substance, they are

prone to revert to the "normal" type, which grows well without a supplement of phosphothiamine. Such normal variants can also be obtained by cultivation in broth containing suboptimal quantities of the coenzyme, or by plating massive inocula (i.e., a billion or more cells) upon the deficient base medium. Each of these methods yields variants completely independent of added phosphothiamine. Moreover, colonies of such variants, as well as of other normal strains of gonococcus, stimulate satellite growth of the deficient strains. It seems likely, therefore, that these variants have acquired the capacity for phosphorylation of thiamine. Certain lines of evidence, to be presented elsewhere, indicate that the deficient strains give rise to occasional "mutant" cells which are selected by conditions unfavorable to the survival of the deficient parent cells. The ratio of these variant cells to the deficient parent cells is so low (perhaps 1 per billion) that they are completely submerged in a culture population for which an ample supply of phosphothiamine is available. Only when the supply is limited do they become a recognizable and dominant part of the culture.

When these fastidious strains are grown upon a medium containing a suboptimal quantity of cocarboxylase they exhibit various morphological aberrations. The cells vary widely in size, with large, swollen cells predominating, although minute forms may be present. Many cells show a tendency to retain the gram stain, but on the other hand, poorly stained or vacuolated "ring" forms are numerous. A striking characteristic is the pronounced tendency to form tetrads and sarcina groups, which, with the large cells and the partial retention of crystal violet, often presents a picture strikingly similar to that of *Gaffkya tetragena* or *Sarcina lutea*. Such deviations in morphology are most marked when the quantity of cocarboxylase is just sufficient for growth, and decreases progressively as the concentration increases. When transplanted to a well-supplemented medium, these atypical forms quickly revert to the normal morphology.

#### DISCUSSION

Few instances have been reported in which microorganisms show preferential utilization of coenzymes as compared with their constituent vitamin moieties. For *Streptococcus salivarius*, Niven and Smiley (18) found cocarboxylase to be 40% more active than thiamine,



while Sarett and Cheldelin (19) observed 30% better growth response of *L. fermenti* to cocarboxylase up to 18 hours incubation, although at 24 hours thiamine was equally effective in equimolecular amounts. Examples of obligatory utilization of coenzymes are at present limited to the codehydrogenase I requirement of *Hemophilus influenzae* and *H. parainfluenzae*. This requirement is not absolute, however, since Gingrich and Schlenk (20) have demonstrated that nicotinamide riboside and codehydrogenase II may be utilized, although far less efficiently. The dihydrocodehydrogenase I was found to be equally as effective as the oxidized coenzyme, whereas the artificial derivative, desaminocodehydrogenase I possessed about 60% activity.

The cocarboxylase requirements of these deficient gonococci appear to be related to some failure in their mechanism for phosphorylation of thiamine. Weil-Malherbe (14) and Lipton and Elvehjem (21) have presented evidence that adenosine triphosphate is the immediate phosphate donor involved in the phosphorylation of thiamine by yeast. It was postulated by the former investigator that the pyrophosphate group is transferred *in toto* to free thiamine, and that the monophosphate is not an intermediate in the synthesis of cocarboxylase. Moreover, Weil-Malherbe suggests that thiamine monophosphate must be hydrolyzed first to thiamine before it can be phosphorylated to cocarboxylase. The evidence obtained in this study suggests that the gonococcus, at least, effects a two-stage phosphorylation of thiamine. The inability of the fastidious gonococci to phosphorylate thiamine appears to apply only to the initial stage of the process, since thiamine monophosphate is roughly 80% as active as the diphosphate. While it is conceivable that monophosphothiamine may function as such (or rather in association with the apoenzyme), it is considerably more likely that it is converted to diphosphothiamine, as this is the only compound known to possess cocarboxylase activity. Whether two different phosphorylases are involved in conversion of thiamine to thiamine diphosphate by the gonococcus (in which case one is lacking in the exacting strains), or whether the usual phosphorylase is present but "defective" to the extent that it cannot initiate the process, can only be speculated upon with the information at hand.

One of the most puzzling aspects of the problem is the fact that the thiazole component of thiamine exhibits roughly one millionth the activity of cocarboxylase, whereas presumably pure thiamine (samples

B<sub>1</sub>C and B<sub>1</sub>D) shows no trace of growth stimulation unless the thiazole is released by cleavage. No less perplexing, however, is the nature of the active "contaminating substance" in some of the thiamine samples. Although the most effective thiamine (B<sub>1</sub>N) is approximately 1/10,000th as potent as cocarboxylase, the active substance itself must possess considerably greater activity, if it be assumed that the active factor constitutes a relatively small portion of the thiamine sample. Moreover, the incomplete destruction of the activity of yeast extract by takadiastase suggests the possible existence of an active natural substance other than cocarboxylase or monophosphothiamine.

Although no attempt has been made to adapt the phosphothiamine requirements of the gonococcus to an assay method for cocarboxylase, such a possibility suggests itself. The obvious disadvantages of such a test organism and the lack of precision of the colony diameter method of estimating growth response are significant handicaps to such an assay. Moreover, these organisms respond similarly to both mono- and diphosphothiamine. The most serious objection, however, is the possibility of the natural occurrence of the unknown component of the active thiamine samples, which would render the test nonspecific. Possibly this last objection might be overcome by using phosphatase-treated controls, since the thiamine "contaminant" is unaffected by takadiastase, in contrast to the labile phosphothiamines. On the other hand, such a test might be useful to detect small quantities of cocarboxylase for which the thiochrome and yeast assays are insufficiently sensitive. It is generally considered, for example, that cocarboxylase is normally absent from urine (22, 23, 24), yet the results in Table IV indicate .1 to .15  $\gamma$  phosphothiamine per 100 ml., although of course this may be actually the monophosphate. No trace was detected in human plasma, however, in accord with previous findings (22), and the value for whole blood is within the accepted range (22, 23, 25). The values of cocarboxylase in tissue extracts are low (26, 27) but no attempt was made to obtain the tissues before autolysis occurred. Moreover, it is quite likely that extraction of the tissues was far from complete. No recovery tests were run, as the absolute value of cocarboxylase was not in question.

The synthesis of cocarboxylase by yeast and bacteria has been demonstrated (28, 29), but it has been suggested that the amount produced is just sufficient to saturate the apoenzyme present in the cell to form a firmly-bound protein-coenzyme complex (14, 17, 28).

It appears that this problem might be reinvestigated with profit, however, in view of the strong satellite stimulation (Fig. 2) of deficient gonococci by certain contaminating microorganisms. This observation suggests that phosphothiamine in considerable excess over the immediate needs of the cells is produced and diffuses into the medium.

Finally, the necessity for presynthesized phosphothiamine as a growth factor for these organisms fails to support the stated hypothesis that thiamine is attached to the apoenzyme before phosphorylation occurs (17).

#### ACKNOWLEDGMENT

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#### SUMMARY

Certain occasional strains of the gonococcus require for growth a thermostable factor present in yeast, blood, and tissue extracts. The activity of these extracts is destroyed by alkali, acid sulfite, and takadiastase.

Cocarboxylase will substitute completely for the natural materials. Maximum colony diameter is produced by  $0.2 \gamma$  %, and a trace of growth may be detected with as little as  $0.002 \gamma$  %. Thiamine monophosphate shows about 80% of the activity of cocarboxylase. Within these limits, and under the proper conditions of culture, the colony diameter of the gonococcus is directly related to the concentration of phosphothiamine.

Certain samples of thiamine possess growth-promoting activity when present in high concentration, whereas other samples are devoid of such effect. In general, the older samples are the most potent. The more common oxidation and cleavage products of thiamine have little or no activity.

Thiamine competitively inhibits the utilization of cocarboxylase by these organisms.

Under the proper conditions of culture, these cocarboxylase-deficient strains give rise to variants which are identical with the "normal" type of gonococcus and which appear to synthesize phosphothiamine.

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# Oxidation of Acetyl Phosphate and Other Substrates by *Micrococcus lysodeikticus*

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## INTRODUCTION

*Micrococcus lysodeikticus* is useful in the study of oxidation of carbohydrates since at least two preparations can be obtained from this organism which are oxidatively active, yet circumvent permeability difficulties encountered in work with intact cells. One preparation is secured by lysis of the cells, *e.g.*, by saliva or egg white, containing lysozyme (7); the second preparation is obtained by treating cells with acetone (8). Lysed preparations are permeable to substances such as oxalacetate and retain the capacity to oxidize several important carbohydrate intermediates, *e.g.*, pyruvate and fumarate (9). Acetone-treated cells are more permeable than untreated cells. Both preparations have the disadvantage that they do not oxidize certain substances (21) readily attacked by intact cells, *e.g.*, succinate and acetate.

Our experiments demonstrate that the oxidative powers of lysed preparations are greatly enhanced by additions of NaCl. After such additions, the properties of the lysed cells are similar to those of intact cells. Acetyl phosphate is oxidized by NaCl-treated lysed cells and a preliminary examination has been made of this substance as a possible intermediate of pyruvate and acetate oxidation.

## METHODS

*M. lysodeikticus* was grown in Roux bottles on a medium containing: 1% glucose, 0.4% yeast extract, 2% agar and 10% tap water. The bottles were heavily inoculated by streaking the entire surface of the agar and then incubated for 72-96 hours at 30°C. The cells were washed off the agar, filtered through glass wool, and washed two or three times with water for fifteen minutes with mechanical shaking. The

cells were then placed in a thin layer on a porous plate and dried *in vacuo*. The dried material was ground lightly in a mortar and the resulting powder stored in the cold for several weeks without appreciable loss of activity. Lysed preparations from dried cells appear similar in all observable characteristics to lysed preparations from undried cells.

At the time of the experiment, the dried cells were washed thoroughly by suspending in water, 100 ml. per g., and shaking vigorously in a mechanical shaker for fifteen minutes. The suspension was centrifuged and the process repeated twice. Lysis was obtained by adding 3 ml. of a 1:20 solution of egg white per g. of dried cells. After addition of the egg white,\* the cells were made up to a final concentration of 80 mg. per ml. by addition of physiological saline. Thirty to fifty minutes were necessary for lysis at 30°C.; the exact time was dependent on the strength of the lysozyme in the egg white solution. Occasional shaking aided lysis. Lysis can be followed roughly by observing the change in color of the preparation; during the process the color of the suspension changes from a bright yellow to a greenish yellow. Changes in viscosity and the production of  $H_2S$  also occur.

Pyruvate was determined manometrically by decarboxylation with ceric sulfate (11). Inorganic phosphate was determined colorimetrically according to Fiske and SubbaRow (6) and acetyl phosphate by the method of Lipmann and Tuttle (16). Acetyl phosphate was used as the lithium salt or as the sodium salt as prepared from the synthetic disilver product.† Adenosine triphosphate was prepared as the sodium salt from the dibarium salt and sodium adenylate by neutralizing a sample of the commercial acid.

The experiments were conducted on the Barcroft-Warburg apparatus at 30.4°C. under conditions described more completely in connection with the individual experiments.

## EXPERIMENTAL

### *Effect of NaCl upon Oxidation by Lysed Cells*

The addition of NaCl to lysed preparations of *M. lysodeikticus* markedly affects the oxidative properties of such preparations. Table I gives a summary of one typical experiment showing the effect of the addition of salt on the oxidation of a number of substrates. Lysed cells were tipped in from the side arm at the end of a forty-minute lysis period. NaCl was absent in the flasks in Column 1, but the lysed cells were added to a mixture containing 0.4 M NaCl in addition to substrate and buffer in Column 2. It should be pointed out that lysis does not cease entirely when the reaction is initiated

\* The egg white was prepared by dilution with physiological saline and was stored for several weeks in the ice box with gradual loss of activity.

† We wish to thank Dr. Fritz Lipmann for the two synthetic salts of acetyl phosphate.

by tipping the lysed cells into the main chamber. Because of the dilution of the lysozyme by the other constituents, the process is considerably retarded. Lysis continues slowly until the cells are almost inactive oxidatively. Possibly the presence of NaCl in the flasks at the time of addition of the lysed cells inhibits further lysis. This point will be discussed more fully in connection with Fig. 1.

In the experiments of Table I, fumarate, pyruvate and lactate were oxidized rapidly without addition of NaCl; succinate,  $\alpha$ -ketoglutarate and, possibly, citrate were oxidized slowly, whereas glucose and acetate appeared to be unoxidized. In all of the above cases, oxidation of a

TABLE I

*Effect of NaCl on Oxidations by Lysed Preparations from M. lysoderivatus*

Expt. No.	Substrate	$\mu$ l. $O_2$ -uptake in 90 minutes	
		No NaCl	0.4 M NaCl
1	None	103	248
2	Acetate	92	412
3	Glucose	128	318
4	Citrate	148	282
5	$\alpha$ -Ketoglutarate	181	424
6	Succinate	219	444
7	Pyruvate	305	540
8	Lactate	330	410
9	Fumarate	415	401

Each flask contained 40 mg. (dry wt.) cells lysed for 40 minutes; 0.06 *M*\* phosphate buffer, pH 6.2, 0.03 *M* substrate (sodium salts in case of acids); water to 2.0 ml. Center well contained 0.3 ml. 20% KOH.

\* Values as final concentrations.

substrate was judged by the increase in  $O_2$ -uptake over the endogenous respiration caused by addition of the substrate. Additions of 0.4 *M* NaCl caused little change in the  $O_2$ -uptake on lactate and fumarate, but stimulated the oxidation of pyruvate. Considerable increases in respiration were noted with succinate,  $\alpha$ -ketoglutarate and especially with acetate. The effect upon the endogenous reaction was large, and it is doubtful whether the increases observed upon additions of NaCl to glucose and citrate were sufficiently large to indicate their oxidation.

It is interesting that the oxidation rates of fumarate,  $\alpha$ -ketoglutarate, lactate and succinate were approximately equal after addition of NaCl. The rapid oxidation of  $\alpha$ -ketoglutarate by the preparation is noteworthy since it is one of the key compounds of the Krebs tricarboxylic



cycle (10). Many species of bacteria and yeasts have been reported to be unable to oxidize this compound rapidly. Citrate, another compound concerned in the cycle, is not actively oxidized, even in the presence of NaCl. However, a number of other tissues have also been reported to be unable to oxidize citrate, at least under certain conditions (2, 23). This deficiency is not, however, a serious objection to the scheme, since recent work with carbon isotopes has indicated that citrate is not a cardinal participant in the cycle (28).

The marked stimulation of acetate oxidation provides an opportunity to study the mechanism of this oxidation with a preparation not involving intact cells, and hence more susceptible to the varied techniques employed in intermediary metabolism studies such as dialysis and enzyme purification.

#### *Effect of NaCl on Pyruvate Oxidation*

Krampitz and Werkman (9) have reported that lysed preparations of *M. lysodeikticus* (without NaCl) oxidize pyruvate to acetate and CO<sub>2</sub> (Reaction 1). Acetate is not further oxidized in completely lysed cells.



This type of respiration has an R.Q. of 2 and an O<sub>2</sub>/pyruvate ratio of 0.5. As shown in Expt. 3 of Table II, these values are closely approached with lysed cells in which no NaCl is added. There is some

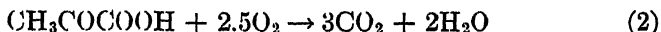
TABLE II  
*Effect of NaCl upon Pyruvate Oxidation by Lysed Cells*

Expt. No.	Additions	O <sub>2</sub> $\mu$ l.	CO <sub>2</sub> $\mu$ l.	R.Q.		Pyruvate utilized $\mu$ l.	O <sub>2</sub> -Pyruvate Ratio	
				Direct	Corrected for Endogenous		Direct	Corrected for Endogenous
1	None	107	108	1.01	—	—	—	—
2	NaCl	246	250	1.02	—	—	—	—
3	Pyruvate	305	556	1.82	(2.26)	511	0.6	(0.39)
4	Pyruvate + NaCl	540	751	1.38	(1.72)	328	1.6	(0.87)

Each flask contained 40 mg. lysed cells and 0.06 *M* phosphate buffer (pH 6.2), in a total volume of 2.0 ml. If indicated, 0.5 *M* NaCl and 0.015 *M* Na pyruvate were added. CO<sub>2</sub> values obtained by the two-cup method. Time, 60 minutes.

uncertainty in the calculation of the R.Q., since it is difficult to determine the proper correction for the endogenous respiration in such experiments. Subtraction of the entire amount of the endogenous values (brackets) results in an R.Q. of 2.26. Although this value is probably not correct, unless an anaerobic decarboxylation was taking place, it is not satisfactory to disregard the endogenous entirely as has been done in the other R.Q. figures. The true correction probably lies somewhere between these two extremes.

Addition of NaCl to the pyruvate oxidation would be expected to change the R.Q. since the previous experiment has shown that acetate is oxidized by NaCl-treated preparations. If there is a comparable effect upon pyruvate oxidation, the reaction should proceed according to Reaction 2.



In this oxidation, the R.Q. is 1.2 and the  $\text{O}_2$ /pyruvate ratio, 2.5. The shift in R.Q. and  $\text{O}_2$ /pyruvate ratio caused by addition of NaCl (Expt. 4) is in this direction and indicates that the oxidation is approaching the course illustrated by Reaction 2.

According to Expts. 1 and 2 of Table II, the endogenous respiration has an R.Q. of 1.0, either in the presence or absence of NaCl. This value suggests that glucose or a polysaccharide is the substrate of the endogenous respiration.

#### *Nature of the Salt Effect*

The exact nature of the effect caused by addition of NaCl is not clear. Upon addition, a visible precipitate can often be seen to form in the manometer flask. It is possible that this aggregating action furnishes a surface for previously dispersed enzymes to come into contact with each other or with substrates and coenzymes, and in that way promotes oxidation. Another factor which must be considered is the possible cessation or inhibition of the lysing process upon addition of NaCl because of inactivation of lysozyme. The lysing process ordinarily continues until a gel is formed (60–80 minutes) and the preparation finally becomes inactive. The addition of salt must be made before the gel formation occurs if stimulation is to result. The NaCl undoubtedly retards this process, but a consideration of the experiments of Fig. 1, makes it unlikely that an inhibition of lysozyme is solely responsible for the salt activation.

If the only action of NaCl is to prevent further lysis, the rate of oxidation following NaCl addition can not be greater than the rate preceding the addition of the NaCl. On the other hand, if NaCl has an activating effect upon the lysed preparation, an increase in rate should occur upon addition of salt to the preparation. That such an increase occurs is shown in Fig. 1. In this experiment, *M. lysodeikticus*

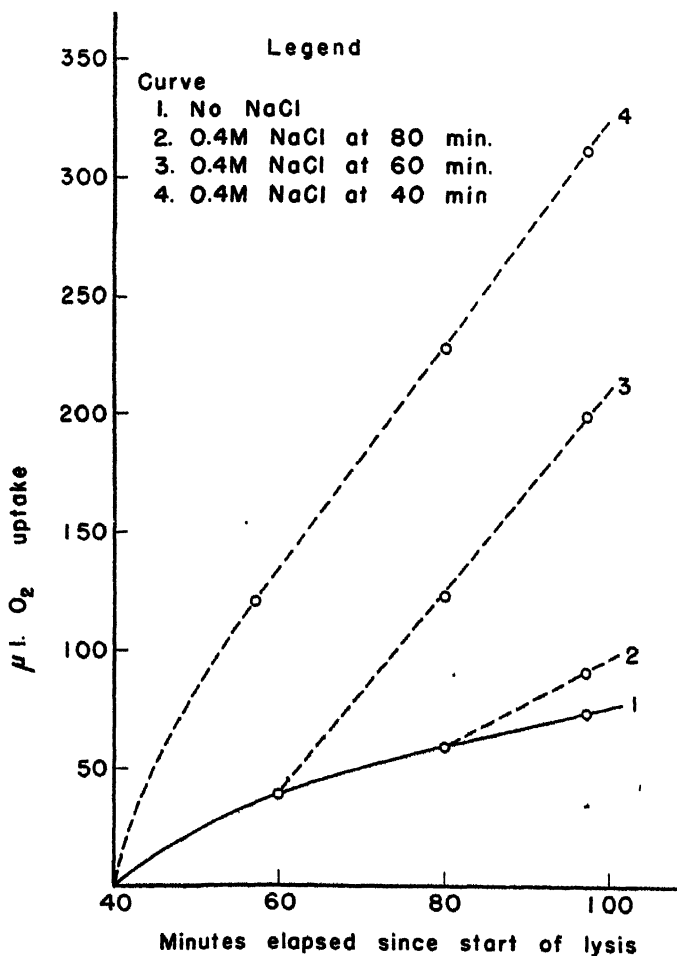


FIG. 1

Effect of Time of Lysis on Stimulation of Oxidation of Acetate by NaCl

was lysed in the usual way and tested for oxidation upon acetate. Readings were started at 40 minutes by tipping in the lysed cells from the side arm of the manometer vessel. The oxidative rate of such a preparation is shown by the solid line of Curve 1. The much higher rate exhibited by a NaCl-fortified preparation is shown in Curve 4. Here the NaCl was tipped in at 40 minutes along with the lysed cells. In a duplicate cup, represented by Curve 3, NaCl was tipped in from a second side-arm after 60 minutes, *i.e.*, after 20 additional minutes of lysis. If the sole effect of the NaCl added at sixty minutes was to stop lysis, the rate should remain unchanged after addition of NaCl. Curve 3 shows that a large change in the rate of  $O_2$ -uptake occurred immediately, and the slope of the line is considerably greater than that of the unfortified preparation (Curve 1) for the previous twenty minutes (40–60 minutes). Thus, it is apparent that a cessation of lysis cannot be solely responsible for the observed NaCl effect. Curve 2 shows the result of NaCl addition after 80 minutes of lysis. In this case, gel formation has occurred and the salt has little effect upon the oxidative rate. It has been observed with more dilute preparations of cells (less than 80 mg./ml.) that the point at which NaCl is ineffective occurs in less than 80 minutes.

The effect of the NaCl is non-specific. Addition of 0.3 ml. of a saturated solution of  $(NH_4)_2SO_4$  in a total volume of 2.0 ml. had essentially the same effect as 0.4–0.6 *M* NaCl.

The most effective concentration of NaCl for stimulation of oxidation varies with the substrate. For example, the optimal stimulation of endogenous and of acetate oxidations occurs at NaCl concentrations of 0.2 and 0.6 *M*, respectively.

#### *Oxidation of Acetyl Phosphate by NaCl-Treated Lysed Cells*

There has been considerable speculation as to the role of acetyl phosphate in pyruvate and acetate oxidation, since Lipmann's demonstration of this compound as an oxidation product of pyruvate by *Lactobacillus delbrückii* (14). In the oxidation of pyruvate by *L. delbrückii*, acetyl phosphate is dephosphorylated to acetic acid but no further oxidation occurs. However, since complete oxidation of pyruvate to  $CO_2$  and  $H_2O$  (Reaction 2) is a well-known reaction of many animal tissues and microorganisms, it is possible acetyl phosphate may be an intermediate in the process. Furthermore, acetyl phosphate

may be an intermediate in the oxidation of acetate, which occurs with certain animal tissues (5) and in some bacteria.

Although acetyl phosphate formation has been reported during two anaerobic dissimilations of pyruvate by bacteria (12, 24), in addition to the aerobic formation by *L. delbrückii*, little light has been shed on its aerobic function. Ochoa, Peters, and Stocken (20) reported that acetyl phosphate failed to increase the  $O_2$ -uptake of brain preparations and also failed to act as a phosphate donor in the same tissue.

We have found that acetyl phosphate is metabolized by NaCl-treated lysed preparations from *M. lysodeikticus*. The labile phosphate can be transferred from acetyl phosphate to an acceptor such as adenylic acid. In addition, there is evidence to indicate that acetyl phosphate is oxidized readily by the preparations.

#### *Transfer of Phosphate from Acetyl Phosphate to Adenylic Acid*

Experiments described in Table III demonstrate that phosphate is readily transferred from acetyl phosphate to adenylic acid. In all of these experiments, 0.4 M NaCl was added to the flask. Determinations of true inorganic P, acetyl phosphate-P, and adenosine triphosphate-P (ATP-P) show the nature of the reaction.

TABLE III

#### *Transfer of Phosphate from Acetyl Phosphate to Adenylic Acid*

Expt. No.	Atmosphere	Description	Values in $\mu$ l. or Equivalents			
			$O_2$	Inorganic P	ATP-P	Acetyl Phosphate-P
1	Air	AA*	303	0	0	
2	Air	Acetyl $PO_4$	158	+130	+30	-170
3	Air	Acetyl $PO_4$ + AA	507	+120	+185	-310
4	Air	Acetyl $PO_4$ (No enzyme)	0	+140	0	-130
5	N <sub>2</sub>	Acetyl $PO_4$	—	+120	0	-120
6	N <sub>2</sub>	Acetyl $PO_4$ + AA	—	+110	+80	-195
7	N <sub>2</sub>	Acetyl $PO_4$ (No enzyme)	—	+140	0	-110

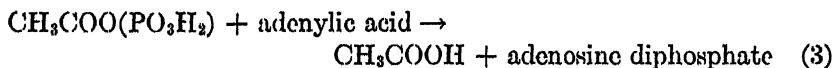
\* AA = adenylic acid.

Each flask contained 40 mg. lysed cells, 0.03 M phosphate buffer (pH 6.2) and 0.4 M NaCl in a total volume of 2.0 ml. If indicated: 224  $\mu$ l adenylic acid, 330  $\mu$ l acetyl  $PO_4$ . Reaction time was 90 minutes.

In all experiments of Table III, including 4 and 7, in which no enzyme was added, a considerable hydrolysis of acetyl phosphate to acetic acid and phosphoric acid occurred. This was to be expected as the compound is very labile. The inorganic P liberated during the experiment, plus the ATP-P formed, should equal the acetyl phosphate-P decrease, if no other phosphate changes involving any of these compounds occurred. This condition of balance holds fairly well in all experiments of Table III.

In Expts. 1-4, the reactions were carried out in air and the  $O_2$ -uptake was measured. When acetyl phosphate was added to the preparation (Expts. 2 and 3), the  $O_2$ -uptake increased over the endogenous respiration (Expt. 1). In Expts. 5-7, the reactions took place in an atmosphere of nitrogen.

When acetyl phosphate was added without a phosphate acceptor (Expts. 2 and 5), little or no ATP-P was formed. However, when adenylic acid was included in the reaction mixture (Expts. 3 and 6), a considerable quantity of ATP-P accumulated. Several experiments similar to the one reported in Table III have all indicated that the transfer of phosphate to adenylic acid is more rapid in air than in nitrogen. It is possible that air (presumably oxygen) merely aids in the phosphate transfer by participating in the removal of the acetate, one of the products of the reaction. However, preliminary experiments involving the addition of acetate to the reaction mixture have indicated that acetate does not influence the transfer in any way. This finding makes the above explanation unlikely. We are giving consideration to the idea that acetyl phosphate undergoes some reaction, for example, a condensation, prior to the transfer of the phosphate, and that this reaction or a subsequent one is influenced by oxygen (1). If the transfer takes place by the mechanism shown in Reaction 3, it is difficult to see how the presence of oxygen could influence the reaction unless the actual activity of the transfer enzyme is affected.



Reaction 3 occurs readily under anaerobic conditions in preparations from *E. coli* (24) and from *M. lysodeikticus* (Expt. 2, Table III), but probably an additional reaction occurs aerobically, at least in the latter case.

*Oxidation of Acetyl Phosphate by M. lysodeikticus*

An increase in  $O_2$ -uptake was observed when acetyl phosphate was added to the manometer flask (Table III). It seemed possible that such an increase in respiration might be an artifact. Therefore, parallel determinations of acetic acid and acetyl phosphate were made (Table IV). In this determination, acetyl phosphate was hydrolyzed to acetic acid, so that both acetic acid and acetyl phosphate were included

TABLE IV  
*Disappearance of Acid During Acetyl Phosphate Oxidation*

Expt. No.	Additions	$O_2$ in $\mu$ l.		Values in $\mu$ l. or Equivalents				
		Acetyl $PO_4$	Corresponding Endogenous	Inorg-P	ATP-P	Acetyl $PO_4$ -P	Volatile Acids	$O_2$
1	None	89	161	+ 90	0	-110	0	
2	NaCl	416	226	+100	0	-110	- 85	190
3	NaCl, AA	507	251	+100	+80	-190	-128	256

Each flask contained 40 mg. lysed cells and 0.03 *M* phosphate buffer (pH 6.2) in a total volume of 2.0 ml. If indicated: 0.4 *M* NaCl, 0.005 *M* adenylic acid, 0.012 *M* Li acetyl phosphate. Volatile acids obtained from similar cups containing 10-fold volumes. Time was 90 minutes.

in the volatile acid fraction. In addition, determinations of the various phosphate fractions were included.

The experiments were conducted in 125 ml. respirometer flasks containing a ten-fold volume of the various reactants. At the completion of the reaction, metaphosphoric acid was added to the cups, and the contents were deproteinated by centrifugation. The centrifugate was steam distilled to twelve volumes and the volatile acids titrated. The values have been divided by ten and calculated in terms of microliters to place them on the same basis as the other determinations.

An increase in  $O_2$ -uptake occurred when acetyl phosphate was added (Table IV, experiments 2 and 3) and NaCl was present. The figures in the next column represent the endogenous respiration under comparable conditions. In Expt. 1 of Table IV, the addition of acetyl phosphate caused a drop in the respiration to a level even lower than

the endogenous. This effect has been noted occasionally when acetate or acetyl phosphate was added in the absence of NaCl. Examination of the last column, "Volatile Acids," shows clearly that this fraction, representing acetyl phosphate and acetic acid, decreased when NaCl was present. In the absence of adenylic acid (Expt. 2), the  $O_2$ -uptake increased 190  $\mu$ l. over the endogenous when acetyl phosphate was added, whereas at the same time 85  $\mu$ l. of volatile acids disappeared. These values are close to the theoretical amount of 2  $\mu$ l. of  $O_2$  for each  $\mu$ l. of acetyl phosphate (or acetic acid) oxidized to completion. Addition of adenylic acid (Expt. 3) increased the  $O_2$ -uptake by 256  $\mu$ l. over the endogenous, and the volatile acid fraction decreased by 128  $\mu$ l.

Although this experiment definitely demonstrates a disappearance of acetyl phosphate or acetic acid during the oxidation, it gives no clue as to the mechanism of the oxidation.

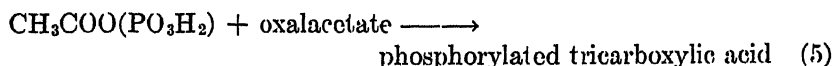
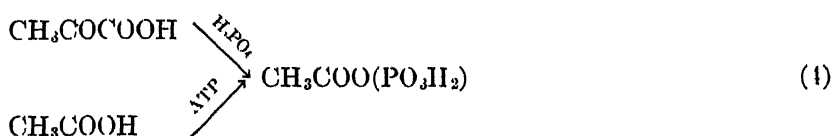
#### *Mechanism of Acetyl Phosphate Oxidation*

There are at least three possible paths by which acetyl phosphate may be oxidized: (a) by dephosphorylation to acetic acid, followed by oxidation of the latter compound; (b) condensation of acetyl phosphate with oxalacetate to form a tricarboxylic acid, as part of a cyclic oxidation process; and (c) conversion of acetyl phosphate to pyruvate by addition of  $CO_2$ , followed by oxidation of pyruvate. A short discussion of each of these possible routes follows.

(a) Dephosphorylation to acetic acid may occur through transfer to adenylic acid (Reaction 3) or through simple hydrolysis to acetic acid and phosphoric acid, either enzymatically or otherwise. Reaction 3 has already been demonstrated in other bacterial preparations (14, 24), and hydrolysis to acetic and phosphoric acids occurs readily. However, certain experimental observations described (Table V) make it doubtful whether this is the main route of acetyl phosphate oxidation.

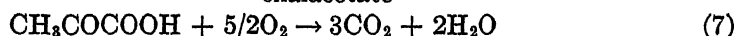
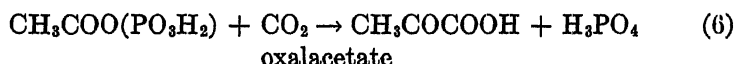
(b) Krebs (10) has pointed out that the experimental evidence bearing on the initial condensation reaction of the tricarboxylic acid cycle does not rule out acetyl phosphate as the possible active intermediate undergoing condensation with oxalacetate. Likewise, acetate might first be converted to acetyl phosphate and then undergo the same reaction, *e.g.*,





The tricarboxylic acid, *e.g.*, a phosphorylated form of *cis*-aconitic acid, might undergo the usual cyclic changes with a loss of the phosphate group at some place in the process. The formation of acetyl phosphate has been reported in three bacterial preparations, but we have been unable to detect any labile P fraction in pyruvate oxidations with *M. lysodeikticus*. However, these experiments are preliminary and negative evidence is of doubtful significance. The transfer of phosphate from ATP to acetic acid has been demonstrated by Lipmann (15). There is no experimental evidence to suggest that reaction 5 occurs.

(c) A third possibility is illustrated by reactions 6 and 7 in which acetyl phosphate is first converted to pyruvate and then oxidized:



A reaction similar to 6 has been demonstrated for *E. coli* (17, 25), in which formic acid combines with acetyl phosphate to give pyruvic acid.

Obviously, it will be extremely difficult to fix the exact path of acetyl phosphate oxidation since the reactions of acetyl phosphate and acetic acid are so closely inter-linked. However, some information is given by the experiments of Table V. As mentioned previously in

TABLE V

*Effect of NaCl Concentration on Oxidation of Acetate and Acetyl Phosphate*

Expt. No.	Substrate	μl. O <sub>2</sub> in 75 Minutes NaCl Concentration		
		0	0.2 M	0.6 M
1	None	85	335	151
2	Acetyl Phosphate	107	441	304
3	Acetate	106	331	420
4	Hydrolyzed Acetyl Phosphate	102	318	454

Each flask contained 40 mg. lysed cells, 0.03 M phosphate buffer (pH 6.2) in a total volume of 2.0 ml. If indicated: 0.018 M Na acetate, 0.013 M acetyl phosphate.

connection with the experiments concerned with NaCl stimulation, the optimum NaCl concentration for stimulation varies with the substrate. Acetyl phosphate and acetate oxidation have widely varying optima. Experiment 1, depicting the endogenous respiration, shows the greatest activity at 0.2 *M* NaCl. Addition of acetyl phosphate shows a similar effect and optimum but when NaCl is present the respiration is increased over the endogenous by addition of acetyl phosphate. On the other hand, acetate oxidation is affected quite differently by the addition of NaCl. At 0.2 *M*, the respiration is little higher than the endogenous but the respiration on acetate is about two and one-half times as large as the endogenous when the NaCl reaches 0.6 *M*. At 0.2 *M* NaCl (according to  $O_2$ -uptake) the acetyl phosphate appears to undergo oxidation but not acetate. It is unlikely that acetyl phosphate oxidation at this NaCl concentration passes through acetate as an intermediate if the latter is not undergoing oxidation.

In Expt. 4, acetyl phosphate was hydrolyzed to acetate and phosphate before the start of the reaction by treatment at an alkaline pH for two hours at room temperature. Addition of the products of hydrolysis gives a result similar to that of acetate itself. Thus, it seems probable that acetyl phosphate in its intact form is necessary to obtain the effect noted in Expt. 2.

Because of the similarity of the endogenous and acetyl phosphate oxidations, it seemed possible that the acetyl phosphate was merely acting to increase the endogenous respiration, at least at a NaCl concentration of 0.2 *M*. Acetyl phosphate might aid oxidation by acting as a source of phosphate energy for phosphorylation of the endogenous substrate, especially since, according to the R.Q. (Table

TABLE VI

*Effect of Adenosine Triphosphate on Endogenous Respiration and on Acetate Oxidation*

Expt. No.	Substrate	ATP Addition 0.0075 <i>M</i>	$\mu$ l. $O_2$ in 90 Minutes NaCl Concentration		
			0.0	0.2 <i>M</i>	0.6 <i>M</i>
1	None	—	95	355	193
2	None	+	121	368	186
3	Acetate	—	116	418	463
4	Acetate	+	135	430	480
5	Li acetyl Phosphate	—	105	520	318

Each flask contained 40 mg. lysed cells and 0.03 *M* phosphate buffer (pH 6.2). If indicated: 0.018 *M* Na acetate, 0.013 *M* Li acetyl phosphate, 0.0075 *M* ATP.

II), this substance appears to be carbohydrate in nature. In the capacity of a phosphate donor ATP should serve as well as acetyl phosphate in stimulating the endogenous respiration. However, ATP (Table VI) has little effect on either the endogenous oxidation or that of acetate at any concentration of NaCl involved in the previous table. Therefore, it is improbable that acetyl phosphate is merely accelerating the endogenous respiration. Lehninger (13) has reported that ATP has no effect on acetate oxidation by liver preparations although the oxidation of higher fatty acids is markedly stimulated.

### DISCUSSION

The oxidation of carbohydrate *via* a tricarboxylic acid cycle is well established in animal tissue but its status in bacterial metabolism is uncertain. The oxidation of pyruvic acid and the four-carbon dicarboxylic acids, and the presence of fumarase have been reported. However, there have been no reports of successful isolation of  $\alpha$ -ketoglutaric acid and citric acids during the oxidation of pyruvate or other crucial experiments to establish the existence of the cycle. Citric acid is dissimilated anaerobically by a few species of bacteria (3, 22), but the relationship of this process to the aerobic metabolism of citric acids is not understood.

Despite the lack of evidence linking bacterial oxidation with the tricarboxylic acid cycle, the recent work of Lynen (18, 19) and Virtanen and Sundman (26) showing a similar cycle in yeast may be cited as a precaution against a premature conclusion that the cycle does not function in bacterial oxidations. Lysed preparations of *M. lysodeikticus*, when fortified with NaCl, rapidly oxidize acetate, acetyl phosphate and pyruvate and, at the same time, cell-free preparations are amenable to the usual techniques employed in intermediary metabolism studies. This preparation should prove useful in extending knowledge of the tricarboxylic acid cycle to bacteria.

The studies involving a condensation of acetoacetic acid with oxalacetic acid in animal tissue to give citric acid or  $\alpha$ -ketoglutaric acid (4, 27), may have a connection with acetyl phosphate metabolism. Although acetyl phosphate has not been detected as yet in animal tissue, it is possible that acetoacetic acid may arise through a condensation involving acetyl phosphate. Actually there is no evidence to eliminate the possibility that acetyl phosphate is the actual inter-

mediate in the condensation and that acetoacetic acid is merely serving as a ready source of acetyl phosphate.

### SUMMARY

1. Addition of NaCl to lysed preparations of *Micrococcus lyso-deikticus* greatly increases respiration on several substrates and permits oxidation of substances which are otherwise not utilized by lysed cells.

2. Lysed cells in the presence of NaCl are able to metabolize acetyl phosphate. Phosphate is transferred from acetyl phosphate to adenylic acid and there is an increased rate of O<sub>2</sub>-uptake and an accompanying decrease in volatile acid.

3. Comparison of the range of NaCl concentrations at which acetate and acetyl phosphate oxidation are stimulated suggests an oxidation path for acetyl phosphate which does not involve acetate as an intermediate. A discussion of the possible routes for the oxidation of acetyl phosphate is presented.

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## Book Reviews

**Vitamins and Hormones, Volume III.** Edited by: ROBERT S. HARRIS, Associate Professor of Nutritional Biochemistry, Massachusetts Institute of Technology, and KENNETH V. THIMANN, Associate Professor of Plant Physiology, Harvard University. Academic Press, Inc., New York, N. Y., 1945, xv + 420 pp. Price \$6.50.

The contents of this valuable volume may be summarized as follows: I. The Interrelation of Vitamins (discussed with respect to animal nutrition), Thomas Moore, 18 pages, 122 refs. II. The Synthesis of B Vitamins by Intestinal Bacteria, V. A. Najjar and R. Barrett, 20 pages, 155 refs. III. Sulfonamides and Vitamin Deficiencies, F. S. Daft and W. H. Schrell, 20 pages, 105 refs. IV. Manifestations of Prenatal Nutritional Deficiency, J. Warkany, 22 pages, 163 refs. V. Growth Factors in Microbiology, B. C. J. G. Knight, 108 pages, >456 refs. VI. Possibilities in the Realm of Synthetic Estrogens, E. C. Dodds, 6 pages, 13 refs. VII. Chemistry of Anti-Pericious Anemia Substances of Liver, Y. SubbaRow, A. B. Hastings and M. Elkin, 55 pages, 129 refs. VIII. Mechanism of Action and Metabolism of Gonadotropic Hormones in the Organism, B. Zondek and F. Sulman, 33 pages, 168 refs. IX. The Role of Acetylcholine in the Mechanism of Nerve Activity, D. Nachmansohn, 37 pages, 131 refs. 42 pages of indexes.

Biochemists in general are deeply indebted to the authors, editors and publishers for their labor in the production of such volumes as this one in which we find summaries of various phases of biochemical investigation. While objectivity, perspective and critical evaluation is not maintained in each chapter with the same degree of excellence, every contribution is valuable. The chapter dealing with synthetic estrogens (of the stilbestrol type) by Dodds is the most restricted as to scope and the references are to work done in the author's laboratory. His frank appraisal that "From the strictly chemical point of view, literally the resemblance (between stilbestrol and natural estrogens) is non-existent" and his reflections upon this fact are valuable.

The most extensive and at the same time a highly coherent review is that of B. C. J. G. Knight, whose early discovery of nicotinic acid as a growth substance paved the way for much of the later work in this field. It is significant of the tremendous advance in this field that this lengthy review is centered around 13 definitely characterized chemical substances or groups of substances, and leaves out of consideration all "factors" of unknown nature. Even folic acid the synthesis of which has in the meantime been announced, is not among the growth substances considered. A few years ago a reviewer on the same subject would have been concerned almost exclusively with unknown factors, and definite chemical substances would have played a minor role in any discussion. Though the author apologizes for not having access to all the literature due to war conditions, he is to be complemented upon the comprehensiveness and relative completeness of his treatment. In one instance the arrival of additional literature from du Vigneaud's laboratory caused him to insert later in proof, pages 228a, b, as a footnote to p. 168. His review deals with a very active field.

Closely related to Knight's review are those of Najjar and Barrett and Daft and Schrell. The three taken together give a large amount of valuable material

dealing with vitamins as they are related to microorganisms and to animals which harbor microorganisms in their intestinal tracts. The two other reviews which deal primarily with vitamins (I and IV) are important in that each has drawn together in one place in a highly satisfactory manner material which is of great importance to every serious student of nutrition.

The discussion of the anti-pernicious anemia substances in liver centers largely around the work of four different groups of workers in their attempts to determine the chemical nature of the active principle or principles. The authors warn the readers, "Unfortunately, within a short time, save for its historical interest, this review may be without scientific value." From the standpoint of the review, this would be unfortunate, but from the standpoint of medical advance, it would be most fortunate indeed. This very difficult subject, the study of which has been continuously hampered by the lack of tests other than those involving afflicted individuals, cannot as yet be reviewed in a satisfactory manner because of the numerous contradictions and discrepancies. The whole subject of intrinsic and extrinsic factors (Castle) is left out of this review and even in the restricted field covered the unexplained discrepancies are many. The probable existence of multiple factors (as well as possibly multiple forms of the disease) is undoubtedly an important factor. The authors are to be complemented on their summary of a subject which is full of perplexities.

The discussion of the action of gonadotropic hormones by Zondek and Sulman covers a rather large amount of older literature and relatively little that is very recent. The authors bring out clearly how different species of animals differ widely in their response to gonadotropic hormones. Unfortunately advance in this field has not been such that definite chemical substances can enter extensively into the discussions, and often investigators must depend on biological responses to materials which are unknown chemically.

The last review in the group has to do with a restricted field in which most biochemists are not well-versed. The author has developed his point of view with respect to the role of acetylcholine: It is "... released at the neuronal surface when a stimulus reaches the nerve cell. By the action of acetylcholine the permeability of the membrane to ions is increased and hence a depolarization occurs. . . . The transmitting agent is the flow of current but the current is generated by the release of acetylcholine." No one interested in neurohumors and nerve physiology should overlook this contribution, since it presents a definite point of view in the light of recent contributions.

ROGER J. WILLIAMS, Austin, Texas

**Die Methoden der Fermentforschung.** Edited by EUGEN BAMANN, Tübingen, and KARL MYRBÄCK, Stockholm. 4 volumes, 3388 pages with 802 illustrations. (Photo offset reproduction.) \$65.00. Academic Press, Inc., Publishers, New York, N. Y., 1945.

To the standard works of chemical world-literature another has been added. This has been accomplished by the above editors. Together with 131 competent specialists they have, in 204 single contributions, summarized most of our knowledge concerning the enzymes and the methods for their isolation, determination and manipulation.

The methods discussed embrace the fields of inorganic, organic and physical chemistry. They are subject to research by chemistry biologists, botanists, zoologists

and medical scientists. They will be applied in the hospital laboratory and the pharmaceutical industry as well as in the laboratories of agricultural research stations and various industrial plants.

The general arrangement of this work is as follows:

Introduction: Nature and effects of enzymes—General consideration of methods in experimental enzymology—Nomenclature and systems of enzymes

Section I: General:

- A. The substrates
  - I. Preparation, properties and investigation of important intermediary and final products
  - II. New methods of investigation in substrate research
- B. The enzymes
  - I. Control of catalysis
  - II. Preparation and analysis of enzymes
  - III. Isolation and characterization of lyo- and desmo-enzymes
  - IV. General procedure for the concentration and separation of enzymes
  - V. Determination of certain general properties of enzymes
  - VI. Appendix

Section II. Theoretical part:

- A. Hydrolysis
- B. Desmolases and biological oxidation and reduction enzymes
- C. Assimilation
- D. Anti-enzymes
- E. Enzyme models

Section III. Practical part:

- A. Enzymes in industry with special consideration of methods preferred industrially
- B. Enzymes in medicine

The contents of this opus are more comprehensive than its title first suggests. In addition to what the title indicates, the work contains most of what should be known of enzyme substrates, thus serving, at the same time, as a useful textbook for the chemistry of substrates. The accuracy and completeness of this work are such that it becomes quite unnecessary to refer to most of the originals. The text may serve as a substitute for a whole library and is therefore indispensable. The subject has been treated thoroughly and completely up to 1940. The bibliography enumerates 7908 titles of publications covered, the names of their authors and references to their places of publication with due accuracy. It is a complete survey of the literature relative to the matter. An outstanding work has been achieved by Bamann and Myrbäck and their collaborators who, in addition to those from Germany and Austria, are from the U.S.A., Great Britain, France, Sweden, Denmark, Holland, Switzerland, Finland, Czechoslovakia, Japan, Italy, and Poland. Considering this opus one is reminded of a statement of Pasteur's: "Scientists have a fatherland, but science itself is international." Because of this spirit, despite the war, this work was completed in the course of the years 1940 and 1941. And thanks



to this team-work, it has remained untouched by the spirit of one-sidedness which prevailed in Germany during those years. The reproduction made by permission of the Alien Property Custodian, is excellent.

CARL NEUBERG, New York, N. Y.

**Manual of the Aspergilli.** By CHARLES THOM AND KENNETH B. RAPER. The Williams and Wilkins Company, Baltimore, Md., 1945. ix and 373 pp. 76 illustrations, 7 color plates. Price \$7.00.

The discovery of Penicillin has brought about an increased interest in molds and demands for broad information to be utilized for wider research as to their possibilities. *Aspergillus* as the name for a genus of molds dates back to 1729 but it was not until the middle of the 19th century that the *Aspergilli* were recognized as agents in many degradations and in a number of industrial fermentations.

The present book is not a monograph and is based upon a comparative study of thousands of strains of *Aspergilli*. It attempts to serve two purposes: (1) to provide the student with means for their identification and to open to him the literature of the group, and (2) to guide the user of the literature in the interpretation of names. The scope of the work can be seen from the following list of the 25 chapters:

Part I. General Discussion	The <i>Aspergillus flavipes</i> Group
Historical Introduction	The <i>Aspergil. versicolor</i> Group
Classification, Generic Diagnosis and Synonymy	The <i>Aspergillus terreus</i> Group
Morphology and Description	The <i>Asp. candidus</i> Group
Cultivation and Examination	The <i>Asp. niger</i> Group
Preservation of Cultures	The <i>Asp. Wentii</i> Group
Variation	The <i>Asp. tamarii</i> Group
	The <i>Asp. flavus-oryzae</i> Group
	The <i>Asp. ochraceus</i> Group
Part II. The Manual Proper	
The Use of the Manual	Part III. Reference Material
The <i>Aspergillus clavatus</i> Group	Topical Bibliography
The <i>Aspergillus glaucus</i> Group	General Bibliography
The <i>Aspergillus fumigatus</i> Group	Check List of Species and Genera
The <i>Aspergillus nidulans</i> Group	Accepted Species, Varieties, and Mutations
The <i>Aspergillus ustus</i> Group	

Thom and Raper brought all the known taxonomic and some of the biochemical material together in an admirable manner. The use of the manual is greatly aided by the inclusion of two types of excellent bibliographies: a general bibliography alphabetical as to author's names and sub-indexed as to date of publication. In addition a chronologically organized topical bibliography is presented.

The omission of two important contributions of recent years was, however, noticed. One by H. Tamiya (*Advances in Enzymology* 2 (1942)), and the other by T. Mann (*Biochem. J.* 38 (1944)). This is especially regrettable in the latter case, since this author pulled no punches and did not hesitate to emphasize that the phosphorus metabolism of *A. niger* differs substantially from processes ascribed to yeast and to tissues.

The volume constitutes a unique reference and laboratory aid, is well produced and is generously illustrated.

F. F. NORD, New York N. Y.

# The Breakdown of Citric Acid in Different Tissues

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Received, January 14, 1946

## INTRODUCTION

In comparative investigations on extensive material, Breusch (1) could show that two groups of tissues exist in warm-blooded animals.

One kind is able to reduce oxaloacetic acid to *l*-malic acid. Their respiration is sensitive even to physiological concentrations of  $\text{Ca}^{++}$  ions. This group includes liver, kidney, muscle, pancreas and, to some extent, brain (probably only grey matter).

The other kind is unable to reduce oxaloacetic acid. Their respiration is insensitive to  $\text{Ca}^{++}$  ions. This group includes tissues of lung, spleen, parotid, placenta, nerve, embryonic muscle and, probably, tumor tissue.

Euler, Adler and Plass (2) and Adler (3) found that the breakdown of citric acid takes place only by means of coenzyme II. Krebs (4) and Breusch (1) found that in this reaction the hydrogen of the citric acid (as compared with isocitric acid) can pass to oxaloacetic acid and is thus reduced to *l*-malic acid.

From this, one can suppose that citric acid can only be metabolized in tissues of the first group but not in tissues of the second group.

In this study evidence is presented that only the organs of the first group are actually able to metabolize citric acid, while tissues of the second group are unable to do so to a measurable degree. That kidney and liver can metabolize citric acid has already been shown by Mårtensson (5) and by Wieland and Jenner (6).

## EXPERIMENTAL

The investigations were carried out with tissues of pigeons, cats, and guinea-pigs. The animals were killed by a blow on the head and

the organs immediately removed and minced in a Latapie mincer. In each experiment 5 g. of tissue pulp was suspended in 20 ml. Ringer phosphate solution (7; page 114). The solution contained 10 mg. of neutralized citric acid and 2 mg. of *cis*-aconitic acid anhydride dissolved and neutralized 2 minutes before the beginning of the experiment. Pure citric acid cannot be used, as 20% of it disappears by the action of the enzyme aconitase, which produces an equilibrium of isocitric acid (16%)  $\rightleftharpoons$  *cis*-aconitic acid (4%)  $\rightleftharpoons$  citric acid (80%). The correctness of this supposition is shown by anaerobic control experiments with minced lung. In a mixture of the above mentioned composition containing much aconitase, the amount of analyzable citric acid remained stable for an hour.

The mixture was shaken aerobically in 250 ml. Erlenmeyer flasks in a water thermostat at 38°C. Shaking took place twice per second with an amplitude of 10 cm. At the beginning of the experiment, after 15, 30 and 45 minutes, 5 ml. of the mixture were pipetted into 5 ml. of 20% trichloroacetic acid. Citric acid was determined colorimetrically according to Pucher, Sherman and Vickery (8). Without the addition of citric acid none of the tissues presented any measurable positive

For 100 g. of wet tissue	Citric acid found after				Citric acid disappearing per hour	Capacity of reduction of oxaloacetic acid per 100 g. of wet tissue/hour
	0	15	30	45		
	minutes					
Liver, cat	208 mg.	6 mg.	2 mg.	2 mg.	808 mg.	1500 mg.
Liver, guinea pig	210 mg.	24 mg.	6 mg.	2 mg.	744 mg.	1200 mg.
Liver, pigeon	192 mg.	7 mg.	6 mg.	1 mg.	740 mg.	1600 mg.
Muscle, pigeon	215 mg.	98 mg.	15 mg.	40 mg.	468 mg.	2400 mg.
Muscle, pigeon heart	188 mg.	30 mg.	7 mg.	6 mg.	632 mg.	1100 mg.
Kidney, cat	215 mg.	115 mg.	40 mg.	20 mg.	400 mg.	2000 mg.
Pancreas, cat	212 mg.	110 mg.	70 mg.	60 mg.	404 mg.	600 mg.
Brain, cat	203 mg.	193 mg.	195 mg.	190 mg.	40 mg.	200 mg.
Lung, cat	194 mg.	196 mg.	190 mg.	189 mg.	-	0 mg.
Spleen, cat	185 mg.	187 mg.	181 mg.	182 mg.	--	0 mg.
Parotid, cat	206 mg.	210 mg.	203 mg.	208 mg.	-	0 mg.
Placenta, cat	190 mg.	185 mg.	178 mg.	175 mg.	20 mg.	0 mg.
Embryonic muscle, cat	196 mg.	198 mg.	201 mg.	196 mg.	--	0 mg.
Nerves, peripheral	200 mg.	198 mg.	202 mg.	203 mg.	--	0 mg.

reaction. With organs of the first group, citric acid disappeared, but not with the organs of the second group. In order to obtain still more convincing results, the experiments with lung, spleen and placenta were repeated with 15 g. of tissue, adding only 6 mg. of the acid mixture, the sensitivity of the test thus being increased 6-fold. The results were the same. All values given are averages of several repetitions.

The data are calculated for 100 g. of wet tissue per hour on the basis of the quantities metabolized in the first 15 minutes. In the last column the capacities of the different organs for reducing oxaloacetic acid are given, partly according to (1), partly using unpublished data.

### SUMMARY

The capacity of different tissues of warm blooded animals (cat, pigeon, guinea pig), for metabolizing citric acid, has been investigated.

One group of organs, liver, kidney, muscle, pancreas and, in small amounts, brain, is able to metabolize citric acid.

A second group, lung, spleen, parotid, placenta, nerve and embryonic muscle, is unable to metabolize citric acid.

In all tissues investigated, the capacity for metabolizing citric acid is nearly parallel to the capacity for reducing oxaloacetic acid to L-malic acid, and also parallel with the inhibition of the respiration of different organs by small concentrations of  $\text{Ca}^{++}$  ions, shown previously.

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# The Preparation and Properties of an Amylase Inhibitor of Wheat \*

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## INTRODUCTION

In communications preceding this one, Kneen and Sandstedt (1) reported the presence of an inhibitor of amylase action in wheat and rye which had the ability to retard the dextrinization and saccharification of starch by salivary, pancreatic and certain bacterial amylases. Takadiastase and malt amylase were not affected. It appeared that the inhibitor was a protein.

The present study greatly amplifies the original observation and provides detailed information concerning the purification of the inhibitor. The purified inhibitor, in conformity with earlier views, shows all the characteristics of a simple protein of medium molecular weight.

The wheat inhibitor bears a resemblance to other inhibitors of amylase action but is not identical with them. The inhibitor of Chrzaszcz and Janicki (2) from buckwheat is a water-insoluble protein whose action appears to be due to adsorption. The wheat inhibitor is water-soluble. It can be precipitated with alcohol. Bowman (3) extracted a pancreatic amylase inhibitor from soybeans with ether. Its action is quite likely the result of mechanical coating of the starch so that amylase cannot approach the molecule. Weidenhagen (4) made reference to amylase inhibitors while working with the activator amylokinase, which Waldschmidt-Leitz and Purr (5) had described earlier; but Weidenhagen offered no evidence concerning the identity of the inhibitors. It is possible that the inhibitor from wheat was encountered by him.

\* We wish to express our appreciation to the Chemurgy Project, University of Nebraska, for financial support of this work.

## EXPERIMENTAL

*Preparation and Purification of the Inhibitor*

Two kg. of milled wheat flour were divided into 4 portions and the portions worked into doughballs, employing about 300 ml. of water for each.

Two doughballs were kneaded successively in a liter of water. The sticky remains were transferred to another liter of water, kneaded therein, then transferred to  $\frac{1}{2}$  liter where they were rinsed. The remaining 2 doughballs were kneaded in the same water except that the first liter, which was too thick, was replaced by a fresh portion. The rubbery remains of the doughballs, after rinsing, were discarded.

The various volumes of water employed in the extraction were combined and centrifuged to remove suspended solids. The somewhat opalescent centrifugate was placed in visking sausage casings and pervaporated by means of a fan to 150–200 ml. The solution was then transferred to a beaker and brought to 70°C. and held at that temperature for 5 minutes. During this time the lumps were broken up. After the solution had cooled, absolute alcohol was added until a concentration of 70% was reached. The precipitate which separated was removed by centrifugation and discarded. Absolute alcohol was then added until the concentration reached 90%. The precipitate and supernatant liquor were allowed to stand for 2 hours, after which the liquor was decanted. This final precipitate, which contained the inhibitor, was washed 3 times with absolute alcohol and dried in a vacuum desiccator. The yield was 9 to 10 g. of a white powdery material. This preparation is referred to in this publication as crude inhibitor.

Fractional precipitation of the crude inhibitor was carried out as follows. Five g. of crude inhibitor were worked with 10 ml. of water until solution no longer occurred. The mixture was centrifuged, yielding an insoluble gum and a solution of inhibitor. The gum was washed with 5 ml. of distilled water and then discarded. The wash liquid and the solution of the inhibitor were combined and absolute alcohol added until the concentration reached 50%. The precipitate obtained was discarded after centrifuging. The alcohol concentration was now increased to 60% and 70%, respectively. At each of these concentrations a precipitate was obtained which was removed by centrifugation, washed twice with absolute alcohol, and dried. The precipitate from 70% alcohol was the more potent of the two (about 3 times) and weighed 1.6 g. It is referred to herein as "fractionated inhibitor."

For adsorption, 250 mg. of fractionated inhibitor were dissolved in 13 ml. of pH 5.5 phosphate buffer (0.05 *M* in phosphate). To this solution were added 7.5 ml. of aluminum hydroxide (25 mg. aluminum oxide per ml., pH 5.0). The mixture was allowed to stand at room temperature for 15 minutes and then centrifuged. The supernatant liquid was discarded. The adsorption product was washed twice with 10 ml. portions of distilled water. Elution was carried out with 7.5 ml. of pH 7.5 phosphate buffer (0.06 *M* in phosphate). The final pH was 7. The mixture was allowed to stand for 15 minutes, then centrifuged. The precipitate was washed 3 times with 5 ml. portions of water. The eluate, plus washings, was dialyzed for 7 days against tap water, as dialysis against distilled water caused destruction of the inhibitor. Concentration in a vacuum desiccator yielded 50 mg. of a grey powder.

*Measurement of Inhibitor*

An aliquot of an inhibitor solution to be tested was added to 20 ml. of a 2% starch solution (Merck's "according to Lintner" starch) which was buffered to a pH of 6.6 by means of 0.005 *M* phosphate, made 0.03 *M* in sodium chloride, and the whole diluted to 25 ml. Five ml. of salivary amylase were added while rotating the flask and the time noted. From time to time 1 ml. of the mixture was removed from the flask and added to 5 ml. portions of iodine solution in a small centrifuge tube. The colors obtained were compared with the standard end point according to Sandstedt, Kneen and Blish (6). The time at the proper end point was noted. Usually the concentration of inhibitor was regulated so that a dextrinization time of 30 to 40 minutes was obtained at a temperature of 30°C.

The salivary amylase solution was obtained by diluting human saliva which had been allowed to age for two days in the refrigerator and then centrifuged. Just prior to use aliquots were diluted so that a dextrinization time of 10 to 14 minutes was obtained in the absence of inhibitor.

*Inactivation by Nitrous Acid*

Sixty mg. of fractionated inhibitor preparation were dissolved in 20 ml. of an acetate buffer, pH 4.6. The solution was then divided into two 10 ml. portions. To the first was added 10 ml. of 2 *N* sodium nitrite solution (previously cooled to 0°C.), to the second, 10 ml. of water as a control. The flasks were kept at 0°C. for 30 minutes after which they were warmed to 22°C. (Sometimes controls were allowed to stand for 24 hours.) The pH of each solution was then brought to 6.6 by the addition of 1 *N* sodium hydroxide. The volume was brought to 30 ml., and 5 ml. aliquots were withdrawn from each flask and added to 20 ml. portions of 2% starch solution. Saliva was added to each solution and dextrinization times determined.

*Digestion by Ficin*

A clarified solution of ficin containing 10 mg. of solid per ml. was adjusted to a pH of 5 with a phosphate buffer. To 10 ml. of ficin solution were added 10 mg. of fractionated inhibitor. The solution was allowed to stand for 24 hours in a refrigerator. Little precipitate settled during this time. The pH was then adjusted to 6.6, 5 ml. of the solution were added to the 20 ml. of 2% starch solution, and the dextrinization time determined after the addition of saliva.

## RESULTS

*Preparation and Purification*

The method of preparation used by Kneen and Sandstedt (1) consisted in an extraction with a large volume of water, concentration to small volume and precipitation of the inhibitor in 90% alcohol. This resulted in a preparation containing considerable foreign protein.

For the purification of the crude preparation from alcohol precipitation the following conditions were selected. The inhibitor was



TABLE I  
*Increase in Concentration of Inhibitor through Purification*

Preparation	Mg. solid to give 30 min. dextrinization	Relative increase in concentration of inhibitor effected
Flour	150	—
Crude inhibitor	2.6	58-fold
Fractionated inhibitor	1.3	116-fold
Eluted inhibitor	.2	750-fold

adsorbed from water solution on aluminum hydroxide and eluted with a phosphate buffer. Adsorption was carried out at a pH of 5.0 and the elution at a pH of 7.0. At pH values below 6 the inhibitor could be completely adsorbed by aluminum hydroxide; whereas above 7.0 no adsorption was possible.

The eluate from the phosphate buffer treatment was then dialyzed against tap water until the test for phosphate was no greater than for fresh tap water.

All attempts to crystallize the inhibitor from the dialyzed solution failed, and for further work we were forced to use the amorphous powder obtained by removing the solvent in a vacuum desiccator. In much of the work the solution obtained from the elution was employed directly.

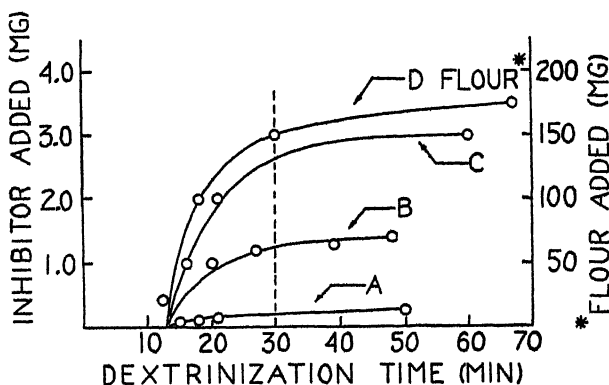


FIG. 1

Inhibiting Powers of Various Inhibitor Preparations  
 "Eluted" inhibitor (Curve A), "fractionated" inhibitor (Curve B),  
 "crude" inhibitor (Curve C), and flour (Curve D)

The amorphous product finally obtained after adsorption and elution represented a 750-fold concentration of the inhibitor from its original concentration in wheat flour. Table I represents values obtained from various fractions of wheat flour subjected to successive stages of purification. The loss of material in each step prohibited continuance of the elution and adsorption beyond the point of a 750-fold concentration.

For comparison of the potency of various preparations of the inhibitor the following method was employed. The quantity of solid preparation was determined which would be required to retard an initial dextrinization time of 12-13 minutes to a dextrinization time of 30 minutes. This was best accomplished by getting the inhibitor-dextrinization curves such as shown in Fig. 1 and interpolating at 30 minutes for the appropriate quantity.

#### *Chemical Nature of the Inhibitor*

The inhibitor preparation of highest potency without question contained protein material, so that routine tests employed, although posi-

TABLE II  
*Inactivation of Inhibitor by Nitrous Acid at 0°C., pH 4.6*

Reaction	Time of reaction	Dextrinization time min.
Inhibitor plus nitrous acid	30 min.	10.5
Inhibitor, no nitrous acid	24 hrs.	30
Nitrous acid, no inhibitor	30 min.	10

tive, indicated little regarding its chemical nature. The inhibitor was thrown out of solution by the addition of such precipitants as lead ion, picric acid and tannic acid; but, as the inhibitor is adsorbed readily, precipitation did not demonstrate a protein nature, since adsorption could readily take place on metal protein complexes. The inhibitor, however, will not dialyze through a cellophane membrane, even on long standing, and therefore it must possess a high molecular weight in line with protein molecules.

The inhibitor was inactivated completely by nitrous acid under mild conditions of pH and temperature which, of themselves, had no effect. Nitrous acid is a standard reagent for the removal of amino groups from protein molecules and has been employed repeatedly for that

specific purpose. In Table II the results of nitrous acid treatment according to the method of Little and Caldwell (7) are summarized.

Groups other than primary amino groups are not affected by nitrous acid during the 30 minute treatment, although the possibility of reaction with the indole ring cannot be ruled out entirely (8). In an effort to relate inactivation to the free amino groups we attempted a determination of the nitrogen evolved with nitrous acid but found that the quantity was not measurable. Such a small amount of nitrogen could only be obtained from a compound similar to a protein in which the ratio of amino groups to molecular weight is very low.

Finally, the activity of the inhibitor was destroyed by the action of proteolytic enzymes. Papain was unable to cause inactivation in 14

TABLE III  
*Inactivation of Inhibitor by Ficin at 0°C., pH 5*

Reaction	Dextrinization time* min.
Digested inhibitor	5
Undigested inhibitor (aged 24 hrs. at pH 5)	120
Control—no inhibitor	5
Control—inhibitor	120

\* To all of these solutions ficin was added during measurement of dextrinizations.

days, but ficin destroyed the inhibitor. Complications due to the presence of pancreatic amylase in trypsin preparations militated against its use, and ficin solely was employed in the studies reported herein as of Table III.

Each of the foregoing points by itself does not prove that the inhibitor is a protein, but when taken together little doubt is left that such is the case. Further evidence is obtained from the studies on heat inactivation.

#### *Stability Toward Dialysis*

During purification of the inhibitor by dialysis, subsequent to elution from alumina with phosphate, it was observed that the solution lost its activity when dialyzed against distilled water. In many cases the activity was completely lost in 4-5 days. If, however, tap water were used in place of distilled, no such inactivation resulted and the solution could be dialyzed free of phosphate without injury. It then developed that, in place of tap water, solutions of sodium chloride or of disodium phosphate could be employed as dialysis media. The data of Table IV illustrate these results.

The loss in activity upon dialysis against distilled water was not due to a change in pH during the process, since the change was never very great. The inactivation

TABLE IV

*Dialysis of Inhibitor Preparation (0.075 mg./ml.) Purified by Adsorption*

Treatment <sup>*</sup>	Dextrinization time min.
Control (no inhibitor)	12.0
Undialyzed eluted inhibitor	23.0
Dialyzed against tap water	23.0
Dialyzed against 0.005 <i>M</i> sodium chloride	23.0
Dialyzed against 0.005 <i>M</i> phosphate buffer (pH 6.5)	22.5
Dialyzed against distilled water	14.0

<sup>\*</sup> Duration of dialysis—12 days.

related to the removal of some dialyzable molecule, presumably inorganic. This is substantiated by the results on dialysis of inhibitor preparations which had not undergone purification by adsorption and elution. Preparations prepared by precipitation with alcohol were not inactivated by dialysis, since they contained sufficient impurities to prevent exhaustion of the protecting element.

The chemical nature of the substance necessary for the stability of the purified inhibitor was not determined.

*Stability Toward Heat and pH*

When compared with other proteins the inhibitor was quite resistant to inactivation by heat but, on prolonged treatment, it was destroyed. Naturally, heat destruction was dependent on pH. At a pH of 5.3 and a temperature of 97–98°C. the inactivation proceeded as shown in Table V.

The curve of inactivation roughly follows a straight line.

TABLE V

*Inactivation of Inhibitor at 97°C., pH 5.3*

Duration of heating min.	Dextrinization time min.
Control (no inhibitor)	15
0	300
5	275
10	255
20	215
30	135
45	90
60	30

Inhibitor concentration at start—1.0 mg./ml.

In more acid solutions and at temperatures below 97°C. the inhibitor is quite stable. Thus, at 65°C. several hours were required to complete the inactivation at a pH of 5.3. Heat denaturation was accelerated by increase in alkalinity, so that at a pH of 9.0 and a temperature of 95°C. inactivation was complete in about 10 minutes. Data covering various pHs and temperatures are given in Table VI.

TABLE VI

*Inactivation of Fractionated Inhibitor at Various pHs and Temperatures*

Temp	Period of heating min	pH						
		5.0	6.0	7.0	7.5	8.0	8.5	9.0
65°	15			28.5	28.5	34.5	31.0	31.0
	30			31.5	32.0	32.0	33.0	27.5
	60			30.0	30.0	30.0	31.5	21.0
	120			30.0	31.0	30.0		
75°	15			41.0	34.5	33.0		
	30			36.0	32.0	25.0	23.5	20.0
	60			32.0	29.5	23.0	17.0	13.0
	120			27.5	24.0	15.0	13.0	13.0
85°	5			31.0	31.0	28.5	30.5	23.5
	10			30.5	30.5	28.0	25.5	18.5
	20			30.5	27.5	22.0	19.0	14.0
	30			27.5	24.5	19.0	16.5	13.5
	60			21.5	16.5	15.5	13.5	13.5
95°	3					21.0	19.0	18.5
	5	40.0	40.5	30.0	24.0	20.5	15.0	11.0
	10			25.5	19.0	16.0	13.0	12.5
	20			20.0	13.0	12.5	12.0	12.5
	30	35.5	34.0	17.0	12.0	12.0	12.0	12.5

\* Figures under pHs are dextrinization times in minutes.

A few of the more illustrative points of this table are as follows. At 65°C. no inactivation of the inhibitor occurred in one hour below a pH of 8.5—a remarkable stability compared to other proteins. At 75°C. and at a pH of 9.0 the inhibitor was completely inactivated in 60 minutes. At 95°C. and a pH of 7.0 the inactivation also occurred in 60 minutes.

*Stability Toward Reducing and Oxidizing Agents*

The object of the present work was to find suitable means of destroying the inhibitor of wheat, so that it would not interfere in the production of alcohol from this grain. Although takadiastase and malt are not affected by the inhibitor, other amylases of possible industrial significance are inhibited or at least are reduced in efficiency. Consequently a number of reducing and oxidizing agents were investigated for their ability to combat the effect of the inhibitor.

The reducing agents employed were hydrogen sulfide, sodium sulfide, sodium sulfite, sodium hypophosphite, hydroquinone and ascorbic acid. The most effective of the list were hydrogen sulfide, sodium sul-

TABLE VII

*Action of Reducing Agents (0.005 M) on the Activity of the Inhibitor (0.07 mg./ml.) at 50°C. for 80 minutes*

	Dextrinization times in minutes pH of treatment		
	5.5	6.0	8.0
Inhibitor	29.0	32.0	37.0
Inhibitor + sodium sulfite	12.5		18.5
Inhibitor + sodium sulfide	23.5		32.0
Inhibitor + sodium hypophosphite	37.5		27.0
Inhibitor + hydroquinone	21.0		29.0
Inhibitor + ascorbic acid		15.0	25.0
No inhibitor*	11.5	15.0	11.5

\* Controls for each reagent showed that excess reducing agent had no retarding action on the amylase activity of saliva during the dextrinization tests at pH 6.6.

fite and ascorbic acid; sodium hypophosphite and hydroquinone were almost without effect. Acidic pHs were more favorable for the inactivation than alkaline. Sodium sulfite was able to cause almost complete inactivation in 30 minutes at 30°C. at pH 6.5 in a concentration of 0.005 M with a solution of inhibitor containing 0.07 mg. per ml. Hydrogen sulfide was less effective at 30°C. than sulfite, but at 98°C. caused a complete destruction within 5 minutes. The inactivation was not reversible. Results are shown in Table VII.

Oxidizing agents were even more effective than reducing agents in destroying the activity of the inhibitor. The reaction, as in reduction, was not reversible. Oxidizing agents investigated were chlorine, bromine, hydrogen peroxide, sodium chlorite, potassium bromate and

potassium chlorate. All except potassium bromate and chlorate were capable of inactivating the inhibitor.

Chlorine, of all the oxidizing agents employed, was the most effective in destroying the inhibitor. The data of Table VIII show that in concentration of 0.00005 *M* chlorine in water, the purified inhibitor (0.07

TABLE VIII

*Effect of Chlorine Water on the Inhibitor (0.07 mg./ml.) at 30°C., pH 5.5*

Chlorine concentration <sup>1</sup>	Duration of action min.	Dextrinization time min.
Control (no inhibitor)	—	11.5
Control (no chlorine)	—	23.0
0.00001	0.5	24.0
0.000025	0.5	17.5
0.00005	0.5	12.0
0.0001	0.5	14.0
0.001	0.5	11.5

<sup>1</sup> Excess chlorine was removed by the addition of 0.01 *M* hydrazine before measuring dextrinization time. Excess hydrazine did not retard the amylase activity of saliva during the dextrinization tests.

mg. per ml.) was almost completely inactivated in 30 seconds at 30°C. The action of chlorine was so rapid that the duration of reaction with the inhibitor was of small importance and the concentration was the more significant. After 0.001 *M* treatment no trace of inhibiting action could be discovered.

The ability of chlorine to cause inactivation was dependent on the

TABLE IX

*Effect of Hydrogen Peroxide (0.01 M) on the Inactivation of Inhibitor (0.07 mg./ml.) at 50°C.*

Duration of heating min.	Dextrinization times (minutes) pH of heating			
	5.5	6.5	7.5	8.5
Control (no inhibitor)*	11.5			
Control (no hydrogen peroxide)	25.0			
5	22.5	21.5	20.5	20.5
15	21.5	18.5	17.5	15.5
20	21.0	18.0	16.0	14.0
30	21.0	17.0	13.0	11.5

\* Excess hydrogen peroxide did not retard the amylase activity of saliva during dextrinization tests.

purity of the sample. If protein impurities were present, a correspondingly greater amount of chlorine was needed. For instance, the addition of 10 mg./ml. of egg albumin to the experimental conditions of Table VIII required a chlorine concentration of 0.01 *M* to effect destruction of the inhibitory action.

All the oxidizing agents were more effective at pHs below 7, except hydrogen peroxide. Its maximum effect was exerted at pHs above 7. From Table IX it will be seen that a 30 minute heating at 50°C. was necessary to inactivate the inhibitor at pH 8.5 with hydrogen peroxide concentrations of 0.01 *M*, whereas at a pH of 5.5 the same treatment had only a slight effect.

### SUMMARY

The amylase inhibitor from wheat was purified by adsorption and elution from aluminum hydroxide. In the process a 750-fold concentration from the original wheat was effected.

The inhibitor was precipitated from solution by protein precipitants, did not dialyze through a viscose membrane, was inactivated by nitrous acid and by digestion with ficin. Hence the conclusion that the inhibitor is a protein.

On dialyzing a solution of the purified inhibitor against distilled water inactivation occurred. This did not occur when dialysis was carried out against tap water, sodium chloride or phosphate buffer.

At alkaline pHs the inhibitor was readily denatured at 95°C. in 10 to 15 minutes. At acid pHs it was much more stable and required more than 60 minutes.

Sodium sulfite, hydrogen sulfide and ascorbic acid inactivated the inhibitor.

Chlorine, bromine, sodium chlorite and hydrogen peroxide caused a destruction of the inhibitor more readily than the reducing agents mentioned in the preceding paragraph.

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# The Mode of Action of an Amylase Inhibitor from Wheat \*

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## INTRODUCTION

In other papers of this series (1) the preparation, occurrence and properties of an amylase inhibitor were reported. The inhibitor is a protein in character and exists in wheat, rye and some sorghums. Only amylases from certain sources are affected by the inhibitors; salivary, pancreatic and certain bacterial amylases are inhibited, whereas malt amylase and takadiastase are not.

In this communication the mode of action is discussed more particularly from the standpoint of kinetics of reaction regarding the inhibition toward salivary amylase. The inhibitor acts in a non-competitive manner toward the amylase in the presence of starch. On this basis one can explain the lack of inhibition for some amylases and the presence of it for others.

## EXPERIMENTAL

### *Determination of Initial Reaction Velocity*

In the uninhibited reaction 1 ml. of stock saliva\*\* solution was added to 4 ml. of .02 *M* phosphate buffer (pH 6.5) and was allowed to incubate exactly 5 minutes in a bath at 30°C. After incubation, 20 ml. of starch solution, 0.03 *M* in sodium chloride, 0.02 *M* in phosphate buffer (pH 6.5) were added by means of a fast flowing pipette. The starch solution was brought to a temperature of 30°C. before addition to the saliva solution. The time required for addition and intimate mixing of the two solutions was about 5 seconds. At the time intervals indicated in Table III, 1 ml. aliquots were withdrawn and the reducing groups formed were determined by the method of Caldwell, Doebbeling, and Manian (2).

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\* We wish to express our appreciation to the Chemurgy Project, University of Nebraska, for financial aid in carrying on this project.

\*\* Human saliva aged for 2 days at 0°C. and clarified by centrifugation.

In the inhibited reactor, 2 ml. of the phosphate buffer were replaced by 2 ml. of "eluted" inhibitor solution. (Inhibitor solution prepared by adsorption and elution from hydrous aluminum oxide.) The inhibiting power of this solution was such that 1 ml. was capable of increasing the dextrinization time\* of 20 ml. of 2% starch (0.125 ml. of stock saliva), from 10 minutes to 40 minutes.

#### *Treatment of Inhibitor with Sodium Cyanide*

Five drops of 10% sodium cyanide solution and 1 drop of 5% sodium nitroprusside solution were added to 35 mg. of inhibitor dissolved in 0.5 ml. of water. This solution was allowed to stand 15 minutes and the development of a pink color noted. A control was run in which the cyanide solution was replaced by an ammonia solution. The pink color which developed in the case of the cyanide-treated solution was not observed with ammonia. Both solutions were diluted with 0.2 M phosphate buffer (pH 6.5) until the inhibitor concentration was 0.07 mg. per ml. One ml. of each of the diluted solutions was then tested for inhibitor. No loss of inhibitor activity was noted in the cyanide treated solution.

#### *Inactivation of Inhibitor with Phenyl Isocyanate*

Seven tenths mg. of phenyl isocyanate were added to 4 ml. of 0.2 M phosphate buffer (pH 6.6) containing 3.5 mg. of inhibitor. The solutions were shaken well and placed in a bath at 30°C. White precipitates separated immediately. After 30 min. the mixtures were removed from the bath, cooled and each extracted with 2.5 ml. portions of ether to remove any excess isocyanate. The white precipitates dissolved completely during the extractions. The pHs of the extracted solutions were 6.3. One ml. of each was diluted with 9 volumes of 0.2 M phosphate buffer (pH 6.5) and the inhibitor in 1 ml. aliquots was determined.

#### *Inactivation of Inhibitor with Aldehydes*

One ml. of inhibitor solution (0.7 mg. per ml.) was added to 9 ml. of 0.2 M phosphate buffer (pH 6.6) containing the appropriate concentration of aldehyde. This solution was placed in a bath (30° or 60°C.) for 30 minutes and then cooled to 25°C. The pH of the solution after heating was still 6.6. One ml. was removed, diluted to 5 ml. with buffer and the inhibitor determined.

## RESULTS

### *Dissociation of the Inhibitor*

During the determination of the strength of inhibitor preparations, it was noted that a newly-prepared solution of inhibitor (from a dry sample) increased its ability to inhibit amylase on standing. For instance, a solution which had an initial action of retarding dextrin-

\* At pH 6.6 and 30°C. in the presence of .03 M sodium chloride in a total volume of 30 ml.

ization from 11.5 minutes to 31 minutes gradually increased its action to 56 minutes after standing for 91 hours at 20°C., as shown in Table I. After sufficient time had elapsed there was no further increase of inhibitory power.

It was found that phosphate and higher temperatures accelerated the development of inhibitory power, so that the conditions finally used to get an inhibitor preparation of maximum power consisted in allowing the solutions to stand for 2 hours at 65°C. No doubt, the action of standing relates to a dissociation of the inhibitor and to the development of active points responsible for the combinations with

TABLE I  
*Effect of Standing on Inhibitor Efficiency*

Time of Standing hrs.	Temperature °C.	Dextrinization Time min.
Control (no inhibitor)	20	11
0.0		31
0.5		32
1.0		36
2.0		35
48.0		54
91.0		56
115.0		56
min.	65	
10		34
30		36
60		50
240		56

pH 6.5; Conc. of Inhibitor 0.07 mg./ml.

amylase. As shown later by kinetic studies, the combination between amylase and inhibitor involves areas on the enzymes which do not enter into the amylase-starch complex.

The combination between amylase and inhibitor is not a reaction which comes to equilibrium immediately upon mixing of the two solutions. The reaction  $E + I \rightleftharpoons EI$  is a reversible reaction and requires an incubation period for the establishment of equilibrium. Kneen and Sandstedt (1) have shown that the amylase can be precipitated in an active state from the complex  $EI$  by the addition of alcohol. In Table II data are presented which show the effect of short periods of incubation prior to the addition of starch on the attainment of

TABLE II

*Effect of Incubation of Inhibitor with Salivary Amylase on Inhibiting Action*

Incubation Time <i>min.</i>	Dextrinization Time <i>min.</i>
Control (no inhibitor)	13
0.1	19
0.25	110
0.5	130
1.0	146
3.0	152
5.0	154

equilibrium as represented by maximum inhibition of dextrinization. An interesting observation concerning an enzyme-inhibitor equilibrium is that no amount of inhibitor can reduce the concentration of the enzyme,  $E$ , to zero and, hence, the reaction catalyzed by the enzyme is never stopped completely. We have found this to be true. Large quantities of the wheat inhibitor, while retarding the dextrinization time enormously, never gave complete inhibition of amylase activity.

*The Reaction Between the Inhibitor and Amylase*

Since the inhibitor-enzyme reaction can be represented by the equilibrium  $E + I \rightleftharpoons EI$ , it follows that the nature of the reaction between the two can be determined by employing the methods already in use for competitive and non-competitive inhibition which have been discussed fully and applied freely (3, 4, 5). In competitive inhibition the substrate of the enzyme and the inhibitor compete for the same reactive points on the enzyme, in a non-competitive inhibition the substrate of the enzyme and the inhibitor occupy different points on the enzyme.

In our work the Lineweaver and Burk treatment (4) was the most applicable, since measurements were made in dilute solutions. For this method, comparison of the results of two equations is necessary; the first from the uninhibited reaction (a), the second from the inhibited reaction (b).

$$\frac{1}{v} = \frac{K_s}{V(S)} + \frac{1}{V} \quad (a)$$

$$\frac{1}{v} = \frac{1}{V} \left[ K_s + \frac{K_s(I)}{KI} \right] \frac{1}{S} + \frac{1}{V} \quad (b)$$

In the equations  $v$  is the initial velocity,  $V$  the maximum velocity,  $S$  the substrate concentration,  $K_s$  the dissociation constant of enzyme substrate complex,  $I$  the concentration of the inhibitor and  $KI$  the dissociation constant of the enzyme inhibitor complex

Equation (b) was derived for competitive inhibition from the consideration  $E + S + I \rightleftharpoons ES + EI$ . If  $1/v$  is plotted against  $1/S$ , straight lines result with various  $S$  concentrations from both the uninhibited and the inhibited reactions which differ from each other in slope. Since  $1/V$ , the maximum velocity, remains the same in both instances, the two lines have a common intercept.

For non-competitive inhibition, (b) is no longer valid. The effect of non-competitive inhibition consists merely in lowering the effective concentration of the enzyme, and hence the maximum velocity  $1/V$  is changed. When  $1/v$  is plotted against  $1/S$  for the uninhibited and inhibited reactions, straight lines are obtained which have identical slopes but different intercepts.

In a more recent publication, Ebersole, Guttentag and Wilson (5) develop the idea of non-competitive inhibition still further by proposing two types for non-competitive reactions. When the inhibitor combines with the enzyme, independent of the presence of the substrate, the equation for the reaction is such that the slope and intercept change upon plotting  $1/v$  against  $1/S$ . In one type the inhibitor attacks both  $E$  and  $ES$  with equal affinity. In a second type the inhibitor attacks  $ES$  with greater affinity than  $E$  and the slope changes less than the intercept.

In the amylase inhibitor our chief interest lay in choosing between competitive and non-competitive inhibition rather than in proving the type of non-competitive inhibition. There is general agreement that a change in intercept of the graph  $1/v$  against  $1/S$  indicates lack of competition by the substrate and inhibitor for the enzyme catalytic point.

The initial velocities of starch degradation by salivary amylase were determined at several substrate concentrations for the uninhibited and for the inhibited reactions. In following starch degradation, saccharifying power was used instead of the usual dextrinizing power (because of its convenience), the method for determining maltose being that of Caldwell, Doebbeling, and Manian (2). To obtain the initial velocity,  $v$ , mg. of maltose were plotted against reaction time in

minutes and the slope of the resulting curves at zero time (as determined by the method of Hanes (6)) yielded  $v$  directly.

When  $1/v$  values were plotted against  $1/S$  for uninhibited and inhibited reactions, the graphs of Fig. 1 resulted. Since the slopes are identical and the intercepts different, the reaction between the wheat inhibitor, the enzyme, and the substrate is a non-competitive reaction.

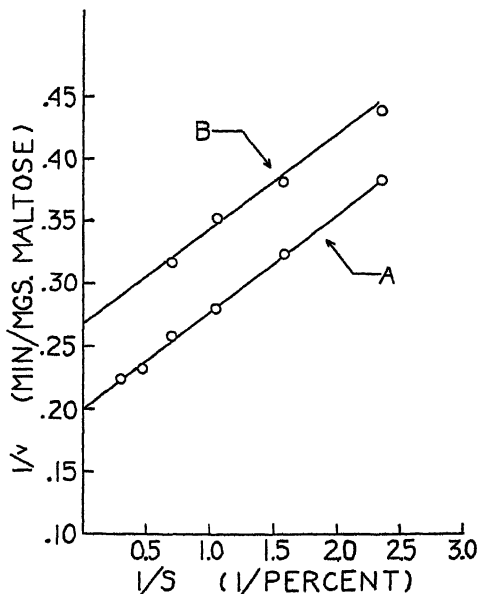


FIG. 1

Reciprocal Initial Velocities ( $1/v$ ) Plotted Against Reciprocal Substrate Concentrations ( $1/S$ ) for the Uninhibited Reaction (Curve A) and for the Inhibited Reaction (Curve B)

This is unusual since most reversible inhibition reactions are competitive.

The non-competitive nature of the inhibition by the wheat amylase inhibitor helps to explain an anomaly which at first seemed difficult of reconciliation with other facts. Not all amylases were inhibited by the inhibitor. Thus, takadiastase and malt amylase were not affected, and pancreatic amylase was affected much less than salivary or bacterial amylase. This difference did not originate from the presence

of a counter-inhibitory substance, since purification of the amylases had no effect on their resistance. If the inhibitor were blocking out the active positions on the enzyme responsible for the splitting of starch (as in a competitive reaction), all amylases should have been affected. Since the reaction is non-competitive it follows that certain amylases have groupings which can combine with the inhibitor while others do not, yet all possess the catalytic groupings necessary for starch hydrolysis.

### *Reactive Groups on Inhibitor*

Destruction of the inhibitor by oxidizing and reducing agents was discussed in a previous paper of this series (1). Since both of these may attack certain groups in the protein molecule, an attempt was made to correlate specific amino acid groupings of the inhibitor with the reagents employed.

Reducing agents such as hydrogen sulfide, thioglycolic acid and sodium bisulfite frequently exert their effect on a protein by reducing the disulfide linkages to sulfhydryl groups. In the active inhibitor no sulfhydryl groups were detectable with the nitroprusside test, but reduction with sodium cyanide yielded positive tests. A correlation between the appearance of sulfhydryl groups and deactivation of the inhibitor was, however, not possible. The reduction by sodium cyanide did not produce inactivation of the inhibitor, and it is most likely that a foreign protein was responsible for the appearance of sulfhydryl groups in the reduction.

The inhibitor preparations gave no tests for tyrosine by the Millon reaction, which simplifies the interpretation of the action of nitrous acid in destroying the inhibitor. The most logical group to be attacked by nitrous acid is the amino group, but this is by no means a certainty since nitrous acid is an oxidizing agent, a nitrosating agent and a nitrating agent. (Tyrosine residues would be attacked readily by nitrous acid.) Phenylisocyanate inactivated the inhibitor, and so it is quite likely that amino groups are involved in the structure—although p-toluenesulfonyl chloride and p-nitrosodimethylaniline did not affect it. The latter may be due to unfavorable solubilities.

Preparations of the inhibitor gave strong tests for tryptophane; and of the amino acids most likely affected by chemical treatment, this one appeared to be most closely linked to the activity of the inhibitor. Halogen and oxidizing agents attack tryptophane with great



case. Further, it was found that aldehydes, such as acetaldehyde, formaldehyde and benzaldehyde, destroyed the inhibitor. The data are shown in Tables III and IV. Of the three aldehydes, benzaldehyde was the most potent in causing inactivation. Acetone had no effect.

TABLE III

*Inactivation of the Inhibitor by Benzaldehyde (0.03 M)*

Time of Action at 30°C. Control (no inhibitor)	Dextrinization Time 12 min.
Inhibitor	30
0.5 min.	24
5	18
10	16
20	15
30	12

Conc. of inhibitor = 0.07 mg./ml.

TABLE IV

*Inactivation of Inhibitor by Aldehydes*

Concentration of Aldehyde	Dextrinization Time Temperature of Standing	
	30°C.	60°C.
Acetaldehyde		
control	11.5 min.	11.5 min.
0.1 M	16	12.5
0.05 M	18	14
0.01 M	22	22
0.00	28	36
Formaldehyde		
control	11.5	11.5
0.1 M	27	14.5
0.05 M	29	14.5
0.01 M	43	20.5
0.00	31	32

Conc. of inhibitor = 0.07 mg./ml.; time of standing = 30 min.

A likely grouping to be attacked by the treatment of proteins with aldehydes is the indole grouping of the tryptophane residues.

Gortner (7) made an extensive study of the reaction between aldehydes and many indole derivatives to yield humins and demonstrated that tryptophane combined with aldehydes during the hydrolysis of proteins. The conditions employed by him were strong acid conditions. The conditions employed in the inactivation of the inhibitor with benzaldehyde (pH 6.5) are mild in comparison, but the

reactivity of the  $\alpha$ -hydrogen of the indole ring should permit a measure of reaction sufficient to denature a protein. Further work is, of course, necessary to demonstrate the point.

### SUMMARY

The effectiveness of an amylase inhibitor obtained from wheat increases upon standing in solution.

The reaction between amylase, starch and the inhibitor is a non-competitive inhibition.

The evidence points to tryptophane of the inhibitor protein as being the sensitive amino acid grouping in the molecule, since reagents known to be specific for this amino acid likewise caused the inhibitor to lose its activity.

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# Fat Formation in *F. Lycoperseci*<sup>1</sup>

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## INTRODUCTION

The most fruitful experimental approach to the problem of the biochemical transformation of carbohydrate to fat has been with certain yeasts, molds and bacteria which rapidly and efficiently synthesize fats from carbohydrate and which are suitable systems for chemical studies. For the purpose of studying this phase of metabolism it was necessary to set up a suitable test system, employing a microorganism, so that the efficiency of intermediates in promoting fat synthesis could be ascertained. Certain strains of *Fusaria* may form from carbohydrate large amounts of a neutral fat (1-4) which is similar to olive oil in composition (3-5). The process may take place in either surface or shaken culture (3). Many of the fungi which have been used in the past in studying transformation of carbohydrate to fat, for example, *Endomyces vernalis* (6), must be grown in surface culture and, hence, would not be suitable for certain types of experiments. *F. lycoperseci* was selected as the test organism after demonstration that this strain could form large amounts of fat from glucose by producing mycelia of 36% fat (dry weight basis) when the fungus was grown on the M solution of Lockwood *et al.* (7).

It seemed necessary at the outset to provide conditions which would separate nonspecific fat formation, occurring as a result of the growth of the mycelia, from metabolic steps which are independent of growth, for it is possible that any substance that could serve for growth might

<sup>1</sup> Supported by grants from the Nutrition Foundation, Inc., and the Sugar Research Foundation, Inc.

<sup>2</sup> Approved by the director of the New York State Agricultural Experiment Station as Journal Paper No. 652, Dec. 1945.

lead to the normal fat formation characteristic of the species. If metabolism could proceed independently of growth, then intermediates might be tested more specifically for their ability to be converted into fat. With few exceptions (8, 9), most workers have ignored this consideration.

The effects of growth and of metabolism may be separated by growing the fungus on a medium in which a low-fat mycelium is produced (growth phase) and then replacing the first nutrient solution by the test medium (metabolic phase) in which there is no source of nitrogen.

In these studies low-fat mycelia were incubated in nitrogen-free solutions which were buffered at pH values from 2 to 9, and the changes in Kjeldahl nitrogen, mat weight and total fatty acids observed. The level of Kjeldahl nitrogen was chosen as a measure of the formation of new protoplasm, since increases in mycelial weight alone may be due only to the storage of fat or carbohydrate. Under the conditions of these experiments it was possible to determine whether growth occurred during the process of fatty acid synthesis and, hence, whether growth processes and fatty acid formation could be separated.

For the purpose of conducting these experiments, a micro titrimetric method based on the procedure outlined by Bloor (10), was also developed for the determination of total fatty acid in the fungus.

## EXPERIMENTAL

### *Micro Titrimetric Method for the Determination of Total Fatty Acids*

The procedure consists in extraction of the lipids from the lyophilized mold by refluxing with boiling alcohol-ether, liberation of the fatty acids, extraction of the latter by petroleum ether, removal of mineral acids and titration of the fatty acids in alcohol solution with 0.02 *N* alkali. The method offers the advantages of speed of extraction of the lipids, adaptability to routine analysis and the use of a small sample. Lyophilization is an essential step in the procedure since unsatisfactory fat extraction was obtained if the fungi were dried by the usual means. The lipid extract must be protected from oxidation during evaporation of solvents by an atmosphere of nitrogen. Petroleum ether was not as satisfactory as alcohol-ether for the direct extraction of fat from the fungus.

With the exception of samples requiring only 0.2 to 0.4 ml. of 0.02 *N* alkali, duplicate analyses checked within  $\pm 6\%$ , a range which is the

same as that obtained by Man and Gildea (11) who present a summary of previous titrimetric methods (12, 13, 14).

### *Method*

The fungus (40–100 mg.) is removed from culture and thoroughly washed with distilled water. The tissue is ground to a fine suspension with a Ten Brock homogenizer (Scientific Glass Co.), transferred to a measuring cylinder and the suspension diluted to 25 ml. Ten ml. aliquots are frozen in tared 50 ml. fat extraction flasks and lyophilized.<sup>3</sup> After determination of the dry weight, the porous residue is extracted by refluxing for 1 hour with 25–30 ml. to a boiling alcohol-ether mixture (3 parts by volume of 95% alcohol to 1 part of freshly prepared peroxide-free ethyl ether).

At the end of this time the solution is filtered through fat-free paper into another fat extraction flask and the residue washed with three 5 ml. portions of alcohol-ether. Two-tenths ml. of 40% potassium hydroxide and a glass bead are added to the flask, the top covered with a close-fitting watch glass and the flask immersed in a water bath at 85–90°C. At the end of 1½ hours the remaining traces of alcohol are removed with a stream of nitrogen, the fatty acids liberated from the soaps by addition of 1.5 ml. of 6 *N* sulfuric acid and subsequently warming until the solid material melts. The flask is allowed to cool, 25 ml. of petroleum ether<sup>4</sup> added and the mixture

TABLE I

*Recovery of Oleic and Stearic Acid Added to Alcohol-Ether Solution<sup>1</sup>*

Acid	Sample No.	Amount added mE	Amount recovered mE	Recovery per cent
Oleic	1 a	0.0656	0.0656	100
	1 b	0.0656	0.0652	99.4
Stearic	2 a	0.0670	0.0670	100
	2 b	0.0670	0.0674	100.5

<sup>1</sup> All titrations were corrected for the blank. Alcoholic solutions of either stearic or oleic acid (Flimer and Amend, CP) were standardized by titration, and aliquots appropriately diluted with alcohol and peroxide-free ether for recovery tests.

refluxed for 10 minutes. The contents of the flask are then cooled, 2 ml. of 5% potassium sulfate solution added and the aqueous layer removed by siphon. Traces of mineral acid are removed by washing the petroleum ether layer with three 3 ml. portions of potassium sulfate solution. The washed petroleum ether is evaporated in a stream of nitrogen at 40–50°C., the residue dissolved in 10 cc. of 95% alcohol and

<sup>3</sup> Satisfactory lyophilization was obtained by placing 12 flasks in a Pyrex 250 mm. desiccator over a fresh charge of 600 ml. of CP sulfuric acid, evacuating to the capacity of a Cenco Hyvac pump, and holding at 5°C. for 48 hours.

<sup>4</sup> The petroleum ether is purified by distillation after standing for 24 hours over sulfuric acid.

the solution brought to the boiling point. After 1 drop of 1% phenolphthalein indicator is added, the fatty acids are titrated with 0.02 *N* alkali while nitrogen is bubbled through the solution. Blank determinations with alcohol-ether solvent are carried out with each set of analyses.

In Table I recoveries of oleic and stearic acids added to alcohol-ether solution are given, and in Table II sample data on the analysis

TABLE II

*Determination of the Total Fatty Acids in the Mycelia of F. lycoperseci*<sup>1, 2</sup>

Sample No.	Weight of Sample mg.	Titration with 0.0211 <i>N</i> alkali ml.	Total fatty acid (as oleic) per cent
1 a	73.9	4.41	35.3
1 b	37.1	2.27	36.1
1 c	37.3	2.37	37.6
2 a	55.0	1.20	12.9
2 b	55.6	1.25	13.4
3 a	31.1	0.275	5.2
3 b	31.3	0.295	5.6

<sup>1</sup> Samples with the same number are aliquots of the same suspension.

<sup>2</sup> All titrations corrected for the blank.

of different mold suspensions covering a wide range of fat content are presented. Oleic and stearic acids were recovered with satisfactory duplication, and proportionate fatty acid recovery using X and 2X amounts of sample provides proof of the adequate extraction of fat from the mycelia.

### *Micro Kjeldahl Determination*<sup>5</sup>

Aliquots of ground mold suspension were digested for one hour with the digestion mixture of Poc and Kalder (15) in which copper sulfate, mercuric oxide and selenium are employed as catalysts. The ammonia was distilled into 2% boric acid and titrated with 0.01 *N* hydrochloric acid using the indicator of Ma and Zuazaga (16).

### *Culture Techniques*

*F. lycoperseci*, obtained from F. L. Wellman, U. S. Dept. Agr. Exp. Sta., Beltsville, Md., Strain No. R-5-6, was maintained on potato-dextrose agars and transferred at monthly intervals. For inoculation of samples a spore suspension was prepared from one agar slant and 50 cc. of sterile water.

<sup>5</sup> A distilling apparatus manufactured by the Scientific Glass Co. (Catalogue No. 3074) was employed.

*Shaken Culture.* 1.5 Liters of medium A,<sup>6</sup> contained in a 3 liter Florence flask, were inoculated with 50 ml. of spore suspension and shaken for 96 hours at 28°C. At the end of this time the mycelia were allowed to settle to the bottom of the flask overnight at 5°C. The supernatant was removed by decantation and the residual mycelia were transferred to sterile centrifuge tubes and rinsed 3 times with 100 ml. portions of sterile distilled water. The washed cells were suspended in 50 ml. of sterile water in a sterile 500 cc. Erlenmeyer flask, the bottom of which was covered with glass beads. By swirling the flask, a fine uniform suspension of cells was obtained. After aliquots of this suspension were removed for fat and nitrogen determinations, other aliquots were transferred to samples of the second medium<sup>7</sup> which were contained in 50 ml. test tubes.

*Stationary Culture.* The low-fat mycelia mats were grown at 28°C. in 125 ml. Erlenmeyer flasks, each containing 20 cc. of Medium A which had been inoculated with 1 ml. of the inoculating suspension. At the end of 96 hours Medium A was removed by pipette and replaced by 10 ml. of Medium B.<sup>8</sup> This procedure was conducted under aseptic conditions and the salts and glucose were autoclaved separately. The chemicals were of the C. P. grade with the exception of the glucose, which was a commercial cerelease.

## RESULTS

The results of experiments in which low-fat mycelia were incubated in shaken and in stationary culture are presented in Tables III and IV. With shaken cultures (Table III) the low-fat mycelium of known fat and nitrogen content was transferred to 16% glucose solutions which were buffered at pH 2 to 8 with phthalic acid-phosphoric acid buffer. The experiments were conducted in triplicate; 7.5 ml. of a glucose solution of a given pH were contained in each of three 50 cc. test tubes which were shaken at 28°C. At the end of 115 hours the contents of the three tubes were combined and analyzed for total fatty acids and Kjeldahl nitrogen. As controls, triplicate experiments were also conducted in which nitrogen (2.0 g. of potassium nitrate per liter) was included in the 16% glucose solution (No. 9, Table III), in which glucose was omitted from the buffer (No. 10, Table III), and in which no inoculating mycelia were added to the 16% glucose solution (No. 11, Table III).

<sup>6</sup> Glucose, 10 g.;  $\text{KH}_2\text{PO}_4$ , 1.0 g.;  $\text{MgSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.5 g.;  $\text{KCl}$ , 0.5g.;  $\text{NH}_4\text{NO}_3$ , 2.0 g. Robbins (17) salt solution, 0.1 cc.; water, 1000 cc.

<sup>7</sup> After the aliquots of mold suspension were added to the aliquots of the second medium, its final composition was glucose, 160 g.;  $\text{N H}_2\text{PO}_4$ , 80 cc., potassium acid phthalate, 1.08 g.,  $\text{N KOH}$  to adjust to desired pH; water, 1000 cc.

<sup>8</sup> Same as Medium A but with a glucose concentration of 25%.



Similar experiments were carried out with stationary cultures (Table IV). The mycelium of known composition was transferred to 25% glucose solutions. These experiments were conducted in duplicate; 10 ml. of a 25% glucose solution that was adjusted to a given pH, were contained in each of two 125 ml. Erlenmeyer flasks. At the end of 163 hours incubation at 28°C., the mycelial mats of each group of two

TABLE III

*Fat Formation in Shaken Cultures*

The cultures were shaken for 115 hours at 28°C. and the fatty acid content calculated as oleic acid

Solution No	Initial pH	Final pH	Mycelium per 100 cc	Percent fat	Fat per 100 cc	Percent nitrogen	Nitrogen per 100 cc	Protein per 100 cc.	Carbohydrate per 100 cc.	Fat nitrogen ratio
			mg	per cent	mg	per cent	mg	mg	mg	
1 <sup>1</sup>	—	—	267	2.5	6	6.97	18.6	116	148	0.32
2	2.1	2.1	250	3.5	9	5.52	13.8	86	155	0.65
3	3.0	3.0	351	6.6	23	4.26	14.9	93	235	1.54
4	3.3	3.2	351	5.7	20	4.44	15.6	98	233	1.28
5	6.0	5.0	443	12.0	53	2.98	13.2	82	308	4.01
6	7.2	6.1	484	10.6	51	2.97	14.4	90	343	3.54
7	7.0	6.0	511	14.3	72	2.87	14.6	91	348	4.93
8	8.0	6.1	477	13.5	64	2.89	13.8	86	327	4.63
9 <sup>1</sup>	3.2	5.4	710	11.0	78	3.82	27.1	169	463	2.89
10 <sup>1</sup>	3.2	4.8	274	1.6	4	4.13	11.3	71	196	0.35
11 <sup>6</sup>	8.0	7.1								

<sup>1</sup> Analysis of mycelia before transfer.

<sup>2</sup> Mycelial weight minus fat + protein contents

<sup>3</sup> 2.0 g. KNO<sub>3</sub> per liter.

<sup>4</sup> No glucose present.

<sup>5</sup> No inoculating mold added

flasks were combined and analyzed for total fatty acid and Kjeldahl nitrogen. As control, 2.0 g. of KNO<sub>3</sub> per liter was added to the 25% glucose solution in two flasks (No. 4, Table IV), and the mats in one group of flasks (No. 1 b, Table IV) were not transferred to the nitrogen-free high glucose solution but left for 163 hours more in the solution in which they were grown. In another series of experiments not included in Table IV, qualitatively similar results were obtained with an incubation period of 115 hours.

The amount of protein in the mycelium was calculated by multiplying the nitrogen content by 6.25; the level of carbohydrate was estimated by subtracting the sum of the amounts of fat and protein from the mycelial weight; the fat:nitrogen ratio was calculated by dividing the mg. of fat by the mg. of nitrogen. The fatty acid was calculated as oleic acid.

TABLE IV

*Fat Formation in Stationary Cultures*

The cultures were incubated at 28°C. for 163 hours and the fatty acid content calculated as oleic acid.

Solution No.	Initial pH	Final pH	Mycelium per 100 cc.	Per cent fat	Fat per 100 cc.	Per cent nitrogen	Nitrogen per 100 cc.	Protein per 100 cc.	Carbohydrate per 100 cc. <sup>5</sup>	Fat:nitrogen ratio
			mg.	per cent	mg.	per cent	mg.	mg	mg.	
1 a <sup>1</sup>	—	7.0	433	15.8	68	5.17	22.4	140	225	3.04
1 b <sup>1</sup>	—	6.8	380	15.6	59	5.50	20.9	131	190	2.82
1 c <sup>2</sup>	—	7.1	335	9.6	32	5.18	17.4	109	194	1.84
2 <sup>3</sup>	5.7	6.1	356	11.2	40	5.14	18.3	114	202	2.18
3 a	2.1	2.0	644	30.2	196	3.98	25.6	160	288	7.65
3 b	2.1	2.0	675	30.8	208	4.34	29.3	183	284	7.10
4 <sup>1</sup>	5.5	6.2	2930	25.6	750	1.65	48.4	303	1877	15.5
5	5.9	5.4	1050	35.4	372	2.39	24.1	151	517	16.1
6	8.4	7.2	1545	24.9	398	1.47	22.7	142	1005	17.5
7	8.7	7.2	1340	25.2	320	1.81	24.3	152	868	13.5
8	9.0	7.1	1330	31.1	413	1.79	23.8	148	769	17.0

<sup>1</sup> Analysis of mycelium before transfer to 25% glucose solution.

<sup>2</sup> Analysis of mycelia which were left in the initial "growth medium" for 163 hours after the other mats were transferred to the 25% glucose solution.

<sup>3</sup> Control solution, no glucose present.

<sup>4</sup> 2.0 g. of KNO<sub>3</sub> per liter added.

<sup>5</sup> Mat weight minus the sum of the fat+protein contents.

Based on the criterion of Kjeldahl nitrogen increase, it appears that little or no "growth" may occur with large increases in mat weight due to deposition of fat and carbohydrate (metabolic phase). At pH 5.9 to 9.0 the protein content of the mycelial mats of stationary cultures increased on an average of about 13% although at the same time the mycelial weight increased 200 to 300%. In shaken cultures (Table III) at the end of 115 hours incubation in a high-glucose solu-

tion, the Kjeldahl nitrogen content actually decreased. It seems possible that the organism in the more vigorously aerated culture was forced to utilize its protein for vital processes. Whenever a source of nitrogen was included in the medium, there was a striking increase in the nitrogen content of the mycelium (No. 4, Table IV; No. 9, Table III). The fat:nitrogen ratio is assumed to be a measure of the efficiency of fat synthesis. From Tables III and IV it is clear that the most efficient fat synthesis, and also the maximum deposition of carbohydrate, occurred at pH 7-8.

### DISCUSSION

The results of these experiments demonstrate that growth processes and fatty acid formation may be separated. With the exception of results with the cell-counting technique of Kleinzeller for *Torulopsis lipofera* (9), these are the first quantitative data of this type. The results should be of value in the interpretation of the data of past experiments on the mechanism of formation of fat from carbohydrate by microorganisms, and in studies now in progress in which the effects of various intermediates on fat synthesis and the probability of metabolic steps in the transformation of carbohydrate to fat will be tested. With this technique the data can be regarded, with some degree of certainty, as indicating a true conversion of tested intermediates to fat, and not simply their availability for growth of the mycelia.

It was found that an initial pH of 7 to 8 was most favorable for fat synthesis from glucose. *F. lycopersci* has a pII optimum for growth at 5.8-6.8 (17) but, in addition, maximum growth has also been observed at pII 4.5 to 5.3 (19), 6.8 (18) and 7.8 (18). Undoubtedly such factors as the composition of the medium and the temperature may account for some of the differences in pII optima which have been reported (20, 21), and it is thus difficult to judge the significance of the finding that the pH optimum for growth is virtually the same as the pH optimum for fat synthesis with minimum growth. The pII optimum for the formation of fat from carbohydrate appears to vary with the microorganism. For example, with *P. havanicum* van Beyma fat synthesis was favored by an acid pH (7); on the other hand, with *Aspergillus fischeri* the optimum range was either neutral or slightly alkaline (22), while fat formation appeared to be independent of pH over a wide range with *Aspergillus niger* (8).

## SUMMARY

A micro titrimetric method for the determination of the total fatty acids in the mycelium has been developed. The synthesis of fat from glucose by *F. lycoperseci* in nonnitrogen-containing solutions has been studied in shaken and stationary cultures which were buffered at different pH values. The greatest synthesis of fat from carbohydrate was observed at pH 7 to 8. Since there was little or no growth of the mycelium (as estimated by the change in the level of Kjeldahl nitrogen) with the formation of large amounts of fat in these high-glucose solutions, the processes of growth and of fatty acid formation have been separated.

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# The Utilization of Amino Acids as a Source of Carbon by Fungi<sup>1</sup>

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## INTRODUCTION

Proteins and their amino acids are the vital constituents of plants, yet only meager information is available on the part which these compounds play in satisfying the carbon requirement of their fungous parasites. It is known that the proteins have a high calorific value and, that under certain conditions, they can furnish energy for growth and respiration. Most of the straight chain amino acids can be assimilated by the animal body and under certain conditions as much as 58% of the ingested protein can be converted to glucose (2). Proteins and peptones are also good media for the growth of some fungi; for instance Klotz (7) grew three fungi on a peptone medium and Krainski showed that both proteins and amino acids can be metabolized by *Actinomyces*. According to Anderson and Emmart leucine, *l*-aspartic acid and *l*-tyrosine are used by *Aspergillus* to produce mycelium. Shade and Thimann (11) showed that *l*-phenylalanine and *d,l*-alanine will support the growth of *Leptothrix*. More recently Dimond and Peltier (3) found that *Aspergillus*, arginine, tryptophane and cystine can be used by *Aspergillus* as a source of carbon. Presumptive evidence for the use of a few amino acids as a source of carbon has been obtained from measurements of respiration (1, 11, 13). Indirect evidence that *Actinomyces* can obtain carbon from proteins and amino acids is also indicated by reports that some of these organisms will utilize less protein when carbon sources are present in the medium (12). Zaleski and

<sup>1</sup> This research was begun while the writer was connected with the Department of Plant Pathology, University of Maryland and was completed while at the Delaware Agricultural Experiment Station, University of Delaware.

Pjukow (11) point out that *Aspergillus* will metabolize more amino acids in the presence of a less available source of carbon than when a more readily assimilated sugar is present.

The purpose of the present investigation was threefold: (1) To study the availability of amino acids as a source of carbon for the growth of fungi, (2) to ascertain whether any correlation could be made between the structures of the compounds and their availability for the production of mycelium, and (3) to determine the effect of their metabolism on the pH of the substrate.

### EXPERIMENTAL PROCEDURE

Two fungi, *Penicillium roquefortii* and *Fusarium oxysporum* var. *lycopersici*, were chosen for these studies, the former because of its ability to grow readily on protein and the latter because it exhibits no such special proclivities. Twenty-one amino acids and four related compounds were tested as carbon sources. Mycelium production was determined in two ways, both in a liquid medium and on a solid medium. The inorganic nutrients were the same as described for Richard's solution,<sup>1</sup> except that amino acids were substituted for the carbohydrate in the original formula at a concentration of 0.5% (10). Wherever possible, the solutions were adjusted to a pH of 6.0 after addition of the amino acids. Some amino acids were insoluble in this range and in those cases, the solution was first made alkaline by adding the amine and then adjusted to the final acidity. Occasionally glucose and the amino acid were incorporated into the same solution to determine whether lack of growth was due to the toxic effect of the amino acid.

Three methods of sterilization were tried; (1) autoclaving the complete medium, (2) autoclaving the medium through a Seitz filter, and (3) autoclaving the basal medium containing only inorganic salts and then adding the amino acids. No differences in mycelial growth were observed and the sterility of the complete medium by passing through a Seitz filter was demonstrated. The latter method was chosen for these studies because of its simplicity.

The initial experiment on the growth of *Aspergillus* and the pH

<sup>1</sup> KNO <sub>3</sub> .....	10	FeCl <sub>3</sub> .....	0.02 g.
KH <sub>2</sub> PO <sub>4</sub> .....	5	Cane Sugar.....	10 g.
MgSO <sub>4</sub> .....	2.5	Distilled Water.....	cc.

determinations were made in liquid media. Five ml. of the medium were pipetted aseptically into sterile test tubes and the tubes were inoculated with the *Fusarium* using a 2 mm. disc of agar, taken from the periphery of a 10-day old petri plate culture of that organism. Spores of the *Penicillium* were obtained from a 14-day test tube culture and aliquot portions of a spore suspension were used to inoculate the amino acid solutions. All solutions were run in triplicate and were shaken twice daily to aerate the media. The extent of growth of mycelium was recorded after seven days and was described by creating six classes, with "0" indicating no growth and "5" as a heavy aerial mat, covering the surface completely. All determinations of hydrogen ion concentration were made with a glass electrode. The mycelium was filtered off after 14 days and the filtrate alone was used for the readings. All pH records are the average of the values obtained from the three replicates.

One fungus, *Fusarium oxysporum* var. *lycopersici*, was used to investigate the relationship between the structure of the amino acids and their utilization as a source of carbon. Because only small amounts of the amino acids were available, growth of the fungus on agar was used as a measure of mycelium production rather than the gravimetric procedure. By this means small quantities of the reagents sufficed to give quantitative measurements. The assay tubes were made by indenting the walls of ordinary bacteriological test tubes, one inch behind the mouth of the tube which prevented the media from escaping when the tubes were placed in a horizontal position.

Two ml. of a 3% agar solution were pipetted into each test tube and the tubes were then plugged with cotton and sterilized. The various synthetic nutrient solutions were prepared at double strength and were passed through a Seitz filter into sterile test tubes. Two ml. of each of these solutions were transferred to the agar tubes, while the agar was still liquid. The medium was inoculated with a disc of mycelium after the agar had set.

Two measurements of growth were used: (1) a linear measurement in millimeters, and (2) a record of the density of mycelium to which values of I and IV were assigned. Preliminary experiments indicated that no correlation existed between linear growth and the density of the aerial hyphae. For example, on the histidine medium the mycelium reached 100 mm. in 14 days and had a density of II, while on the leucine medium it reached only 61 mm. but had a density of IV.



Another experiment with increasing concentrations of dextrose from 0.5 to 5% showed no important differences in linear growth but a definite increase in density was associated with increasing concentrations of sugar. A composite measure of growth was therefore derived by multiplying linear growth and density.

## RESULTS AND CONCLUSIONS

### *Effect of Amino Acids on Growth*

Both *F. oxysporum* var. *lycopersici* and *P. roquefortii* can utilize most of the amino acids for growth (Tables I and II). The linear growth of the *Fusarium* in agar media is a straight line function of time, except for a slight lag during the first twenty-four hours. A similar lag occurs in the dextrose media, which contained the same carbohydrate as the inoculating disc, indicating that this lag phase is not due to the use of amino acids or to a readjustment of the enzyme systems to a different type of medium. The linear growth rate of this fungus over a period of more than twenty-one days showed the absence of any "staling" products of either dextrose or amino acid metabolism.

A quantitative study of the efficiency of the various amino acids in furnishing carbon to the *Fusarium* revealed marked differences in the growth-supporting capacities (Table II). The *Fusarium* produced the greatest amount of mycelium from *d,l*- $\beta$ -phenylalanine, *l*-proline, valine and *l*-hydroxyproline, whose growth-supporting capacities were mostly greater than that of dextrose. On norleucine and *d*-lysine only a trace of mycelium was produced. The other amino acids were intermediate in this respect. The most rapid linear growth occurred on *l*-histidine, valine, norleucine, *d*-lysine and dextrose, while the densest aerial hyphae were produced on *d,l*-phenylalanine, *d,l*-leucine and *l*-proline.

### *Carbon Content of the Amino Acids and Growth*

It is apparent that differences in the arrangement of the carbon atoms in the molecule were not the primary factors in determining the availability of the amino acids as a carbon source. Compounds such as phenylalanine, which contains a carboxylic ring, allowed more rapid growth than the aliphatic compound leucine (Table II). Some of the straight chain aliphatic compounds, in turn, were better than one of the heterocyclic materials, histidine. The percentage of carbon in the molecule appeared to be a more important factor in

determining the availability of an amino acid. When the amino acids were arranged in a series according to their percentage of carbon, and the growth of the *Fusarium* on each amino acid was placed alongside

TABLE I

*Growth of Two Fungi in Amino Acid Media and Their Effect on the pH of the Solution<sup>1</sup>*

Amino Acid	Adjusted pH	Growth of <i>F. oxysporum</i> var. <i>lycopersici</i>	Final pH	Growth of <i>P. toquesfortii</i>	Final pH
Cheek (no carbon)	6.0	None	6.1	None	6.0
<i>d,l</i> -Alanine	6.0	+++	8.1	+++	8.2
$\beta$ -Alanine	5.6	++	8.6	+	8.2
<i>d</i> -Arginine hydrochloride	6.0	++	8.1	+++	8.3
<i>l</i> -Aspartic acid	6.0	++	9.0	++	7.1
Cysteine hydrochloride	6.0		6.0		6.0
*Cystine	6.0		6.0	-	6.0
Dextrose	6.0	+++	6.1	+++	5.9
Glucosamine	6.0	+++	-	++	
<i>d</i> -Glutamic acid	6.0	+++	9.1	++	7.5
Glycine	6.0	+++	8.7	+++	7.8
<i>l</i> -Histidine	6.0	++	6.6	++	6.4
<i>d,l</i> -Leucine	6.3	+++	8.1	+++	8.0
<i>d,l</i> -Methionine	6.0	-	6.7	--	6.3
Nucleic acid	6.7	++	6.5	++	6.3
<i>d,l</i> - $\beta$ -Phenylalanine	6.3	++++	7.5	+++	7.5
<i>l</i> -Proline	5.9	++++	8.1	+	7.5
<i>d,l</i> -Serine	6.0	+++	8.1	+++	7.4
Sodium asparaginate	6.0	+++	8.9	+	5.9
<i>l</i> -Tryptophane	6.0	++	6.9	+++	6.7
* <i>l</i> -Tyrosine	6.3		7.5		7.1
Valine	6.1	+++	6.8	++	6.6
Creatinine	6.0		6.3		6.2
Creatine hydrate	6.0		6.4		6.1
Creatinine+glucose	6.0	+++	5.8	+++	5.7
Creatine hydrate+glucose	6.3	++	6.3	+++	6.0

<sup>1</sup> Classes for production of mycelium range from + to +++++.

\* These compounds were insoluble in Richards solution.

the carbon content, the amount of mycelium produced by the organism generally followed the same order as the percentage of carbon in the molecule (Table II). Some discrepancies, however, did occur. The

amount of growth on tryptophane and leucine was much less than would be expected. On proline it was too great, while growth on glutamic acid was also a bit low. Although the amount of mycelium increased with increasing percentages of carbon, no direct proportionality between the carbon content and the mycelium was evident. These

TABLE II

*Quantitative Studies of Growth of Fusarium Oxysporum var. lycopersici  
with Various Amino Acids as a Source of Carbon*

Amino Acid	Percentage Carbon	Linear Growth in mm.	Density of Mycelium <sup>1</sup>	Composite Rating of Growth <sup>2</sup>
<i>d,l</i> - $\beta$ -Phenylalanine	65.41	86.0	IV	341
<i>l</i> -Tryptophane	64.67	77.7	III	233
<i>d,l</i> -Leucine	54.92	61.7	IV	216
Norleucine	54.92	92.3	tr.	tr.
<i>l</i> -Proline	52.14	82.7	IV	330
Valine	51.24	91.3	III	273
<i>l</i> -Hydroxyproline	45.77	89.3	III	267
<i>d</i> -Glutamic acid	40.80	71.5	III	211
<i>d,l</i> -Alanine	40.42	75.6	III	226
$\beta$ -Alanine	40.42	72.5	III	217
Dextrose	40.01	90.0	III	270
<i>l</i> -Histidine hydrochloride	37.68	100.0	II	200
<i>l</i> -Aspartic acid	36.07	85.0	II	170
<i>d,l</i> -Serine	34.27	80.0	II	160
<i>d</i> -Arginine hydrochloride	34.25	71.3	II	142
<i>d</i> -Lysine dihydrochloride	32.87	91.5	tr.	tr.
Glycine	31.98	70.0	II	140
Sodium asparaginate	31.17	76.0	I	76
Check (no carbon)		0.0	0	0

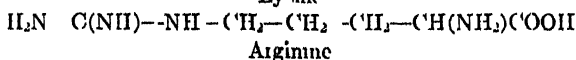
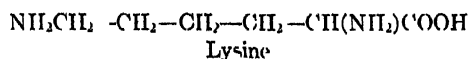
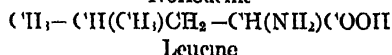
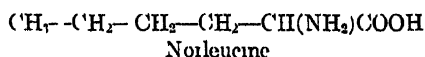
<sup>1</sup> Density was rated from I, denoting a sparse aerial hyphae, to IV, denoting a heavy, almost opaque, aerial mycelium.

<sup>2</sup> Composite ratings were obtained by multiplying linear growth by the density.

discrepancies in order of growth and the lack of proportionality of mycelium to *per cent* carbon might be due to errors in judging density of the mycelium. Another important factor could be the efficiency of the different isomers of an amino acid in supporting growth, since some of the amino acids used were the naturally occurring isomer while others were racemic mixtures.

*Lysine and Norleucine*

The *Fusarium* grew very poorly on lysine and norleucine (Table II). Only a few widely-scattered appressed hyphae were produced, so that the mycelium was barely visible to the naked eye. Nevertheless, the linear growth rate of the hyphae on these acids was very rapid, surpassing that of most other compounds. The scant growth of the fungus on these two amino acids is puzzling, since their structure is relatively simple and is similar to other amino acids which were readily assimilated.



Norleucine is a stereoisomer of leucine and the two compounds differ only in the position of one of the carbon atoms. Norleucine has all six carbons in a straight chain whereas leucine has five of the carbons in a straight chain and the sixth constitutes a side chain. Lysine is believed to be a precursor of arginine (6), an amino acid which supports good growth. It differs from arginine by having six carbons in a straight chain while arginine has only five such carbons. In addition lysine has an amino group on the last carbon, whereas arginine contains a guanidine group in that position. An examination of the structure of norleucine and lysine reveals that they have one feature in common, they are both six-carbon straight chain compounds. It is conceivable that the fungus might not be able to assimilate six carbon straight chain amino acids. Additional evidence for this explanation can be adduced from the studies of Nielsen (9) on the utilization of amino acids by yeast as a source of nitrogen. He reported that *d*-lysine and *d,l*-diaminoadipic acid, which contain six straight chain carbons, were only 1% assimilated by *Saccharomyces cerevisiae* and that *d,l*-diaminopimelic acid, a seven-carbon acid, was not assimilated at all.

The ability of *Fusarium oxysporum* var. *lycopersici* and *P. roquefortii* to grow on so many diverse amino acids might indicate that these

fungi possess a variety of enzymes necessary to carry out the degradations and oxidations of the compounds. Even the ring structures appear to be attacked and furnish carbon for growth and energy. Compounds such as hydroxyproline in which 80% of the carbon is in the pyrrole nucleus are, nevertheless, good sources of carbon. It is not known whether the amino acids are first converted to glucose, as in the animal body (1), and then oxidized, or whether they are oxidized directly as in the case of the fatty acids. In either case the first step could be the hydrolysis of the  $\alpha$  amino group and the substitution of a hydroxyl or keto grouping. Ehrlich (4, 5) showed that in media deficient in any other forms of nitrogen, some fungi will obtain their nitrogen in this manner.

#### *Sulfur-Containing Amino Acids*

Three sulfur-containing amino acids were tested for their ability to furnish carbon for *F. oxysporum* var. *lycopersici* and *P. roquefortii* (Table 1). Methionine dissolved readily in the Richard's solution but cysteine hydrochloride remained in solution only occasionally at a pH of 6.0. Cystine hydrochloride, on the other hand, always came out of solution at a pH above 2.5. Nevertheless cystine was included in the study because fungi sometimes have the ability to solubilize compounds during the process of germination and growth. No growth was observed with any of the three sulfur-containing amino acids in these trials. Six different fungi were then used in similar experiments with methionine alone. They were *Alternaria solani*, *Helminthosporium sativum*, *Rhizoctonia solani*, *Fusarium moniliforme*, *Chaetomium globosum*, and *Aspergillus niger*. None of these fungi produced mycelium in the methionine media. The inability of all 8 fungi to metabolize the sulfur-containing amino acids indicates that the absence of growth was due to the nature of the particular amino acids and not to a chance peculiarity of the organisms originally used. At least two explanations of these results are possible. (1) That methionine and cysteine were toxic to fungi in the concentrations at which they were tested, and (2) that such amino acids cannot be assimilated by some fungi. To determine which explanation was more plausible, a series of experiments was conducted with the 8 fungi in which the methionine media were first inoculated with the organism and incubated for 14 days. Sufficient dextrose was then added to the media to make a concentra-

tion of 0.5% and the solutions were sterilized by means of a Seitz filter. The media were then reinoculated and growth observed. Vigorous mycelium was observed in all tubes on the third day and a heavy mycelial mat was formed by the seventh day. Thus, the inability of the fungus to utilize the sulfur compounds could not have been due to any toxic effect of the amino acids on the fungi unless the dextrose subsequently inactivated the toxin. The results indicated that most probably sulfur-containing amino acids are not metabolized by these organisms.

The lack of any growth in media containing the sulfur-amino acids might be due to the inability of the fungi to split the sulfur-carbon bond, since compounds very similar to those amino acids are readily available to the fungi. Alanine, on which the organisms grow rapidly, resembles cysteine which does not support growth. Both are three-carbon straight chain compounds and differ only in the presence of the sulphydryl group on the  $\beta$  carbon of cysteine. Similarly leucine resembles methionine in having a five-carbon straight chain while it also differs by the presence of a methyl side chain on leucine.

### *Related Compounds*

Creatinine and creatine hydrate, which can be metabolized by *Actinomyces* as a source of nitrogen (12), was not utilized by the *Fusarium* and *Penicillium* for their carbon requirements (Table I). Neither compound is poisonous to the organisms for, when dextrose was added to these materials, the fungus produced an abundant mycelial growth. Glucosamine and nucleic acid both furnished carbon for fungus growth.

### *Amino Acids as Sole Source of Both Carbon and Nitrogen*

Four amino acids and glucosamine were tested to determine whether they could be used by the fungi as the sole source of both carbon and nitrogen. The inorganic nitrogen salt was, therefore, left out of Richard's solution in these experiments. *F. oxysporum* var. *lycopersici* grew rapidly on all five compounds, glycine, valine, aspartic acid, arginine and glucosamine. *P. roquefortii* grew poorly on valine and produced only a trace of mycelium on glycine, while profuse mycelium was produced on the remaining materials.

*Amino Acids and the pH of the Media*

The growth of the two fungi tended to increase the pH of the amino acid media and usually the higher pH values were associated with the media on which more rapid growth occurred (Table I). Increases in pH from 6.0-8.0 were frequent though even more alkaline reactions occurred in solutions of glycine, aspartic acid, glutamic acid and sodium asparaginate. No relationship between the acidity of the amino acid and the final pH of the media was apparent. For example, a greater alkalinity was produced by the *Fusarium* from the acidic compounds, aspartic and glutamic acids, than from the basic compound, arginine, while, with the *Penicillium*, the converse occurred. The metabolism of dextrose by both fungi did not bring about any increase in pH. The production of basic materials in the amino acid solutions is in accord with the investigations of Dimond and Peltier (3) who suggest that the ammonia produced by the hydrolysis of the amino group is not assimilated as rapidly as the acidic elements. One would then expect that solutions of arginine which contains two basic nitrogen groups would become more basic than compounds having only one amine group. However, no such simple relationship seems to exist.

## SUMMARY

Twenty-one amino acids and four related compounds were tested as a source of carbon for the growth of two fungi, *Fusarium oxysporum* var. *lycopersici* and *Penicillium roquefortii*. The fungi utilized seventeen of these compounds for growth.

Very little mycelium was produced from the six-carbon straight chain amino acids, norleucine and lysine.

The sulfur-containing amino acids, cysteine and methionine, did not support the growth of the organisms. The absence of growth was not due to any toxic effect of these amino acids. Creatinine and creatine hydrate also were not metabolized by the fungi.

Glucosamine was a very good source of carbon while nucleic acid furnished only moderate amounts for growth.

The fungi produced an alkaline reaction in the amino acid media.

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# Additional and Corrected Data on the Respiratory and Fermentative Activity of Yeasts Containing Stored Reserves

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In a recent paper in these Archives (Lindegren, 1945, Table II, page 131), I reported on the respiratory and fermentative activity of yeast cells containing visible deposits of fat and glycogen as compared with the activity of cells without visible reserves. Unfortunately, the calculations of the  $Q_{CO_2}^P$  values were found to be in error. However, the original data were still available and were recalculated. The recalculated data appear in Table I. In addition, the corrected value of  $Q_{CO_2}^P$  for culture (155  $\times$  EM1A) is 19 instead of - 41 as shown on page 126.

The yeast for these experiments was obtained by inoculating 50 cc. of nutrient broth containing 1% glucose in 500 cc. Erlenmeyer flasks with a heavy suspension of yeast. About twenty hours later observations showed no budding and no dead cells. Two bakers' yeasts (*S. cerevisiae*, R and U) and a hybrid (*S. cerevisiae* by *S. globosus*) were used. The contents of some Erlenmeyer flasks were poured into 8  $\times$  1 inch tubes to a depth of about 4 inches (30 cc.) and sufficient 60% glucose solution added to make the total concentration 4%. These cultures were grown without shaking to produce conditions favorable for glycogen synthesis (samples 1, 2, and 3, Table I). In a second series, the sugar, sufficient to produce a total of 4%, was added directly to the Erlenmeyer flasks which were then placed on a shaker, to produce conditions favorable for fat synthesis (samples 4, 5, and 6). Tests with Fehling's solution were made and sugar added as necessary to both series. Microscopic examinations were also made to observe the deposition of glycogen and fat. When additional sugar was added, budding began again, indicating that some of the added sugar was used for further growth. After forty-eight hours, budding had ceased and the three yeasts grown in 8  $\times$  1 inch tubes had accumulated glycogen in 100% of the cells. The shaken cultures were all found to contain fat by microscopic observation. The pH of the broth in the shaken culture was 8.1 and of the tubes 4.3. Under heavy aeration the yeasts consume acids; while under anaerobic conditions acids are formed more rapidly than they are consumed.

The respiratory and fermentative activity of the cells containing stored reserves can be compared with the activity of cells without

stored reserves from another experiment. These latter cells were prepared in aerated broth and collected before either glycogen or fat had been deposited (see Table III). The  $Q$  values of these yeasts without visible deposits and with glucose as added nutrient were:  $Q_{O_2}$ , 10–137,  $Q_{CO_2}^O$ , 160–432,  $Q_{CO_2}^N$ , 241–308. The low  $Q_{O_2}$  value probably indicates that this culture contained dead cells.

The percentage of protein was calculated on the basis of the total nitrogen analysis. The cells without visible deposits contained about 55% protein. The cells with visible fat deposits showed approximately the same protein content revealing that they contained relatively little stored fat. However, the protein content of the cells containing

TABLE I  
*Respiratory and Fermentative Activity of Yeast Containing Visible Deposits of Fat and Glycogen*

Visible Deposit	Sample	Culture	Protein	$Q_{O_2}$	$Q_{CO_2}^O$	$Q_{CO_2}^N$	$Q_{O_2}(N)$	$Q_{CO_2}^O(N)$	$Q_{CO_2}^N(N)$
Glycogen	1	R	33.2	0	63	116	0	1178	2187
Glycogen	2	C×GII	38.7	52	156	117	840	2520	1885
Glycogen	3	U	33.5	47	82	83	880	1523	1550
Fat	4	R	56.3	76	249	322	850	2760	3580
Fat	5	C×GII	57.5	127	500	377	1353	5340	4030
Fat	6	U	50.0	125	151	261	1565	1881	3261

visible glycogen was about 35%, indicating a large accumulation of stored glycogen.

The centrifuged cells were washed with  $M/15$  phosphate and placed in Warburg vessels containing 4% glucose. The respiratory and fermentative activity were determined and the figures in Table I show that the accumulation of glycogen caused a considerable decrease in  $Q_{CO_2}^O$ ,  $Q_{O_2}$ , and  $Q_{CO_2}^N$  values. The rather conspicuous fat deposits, however, did not interfere seriously with either fermentation or respiration.

Additional data on respiratory and fermentative activity of cells containing stored reserve material were obtained in the following experiments. A 1% glucose broth was prepared with half the standard amount of nutrient broth to insure the early cessation of growth and more speedy storage of reserve materials. Fifty cc. of broth in 500 cc. Erlenmeyer flasks were inoculated and shaken for forty-eight hours. The suspensions of cells from some Erlenmeyers were placed in  $8 \times 1$  inch tubes to

favor the development of glycogen, while the remainder were kept on the shaker to favor the development of fat. Tests with Fehling's solution were made to determine when the sugar disappeared and sugar was added as soon as a deficiency was indicated. Phosphate was also added, since this is known to increase the deposition of both fat and glycogen. The addition of sugar was continued for four days.

The same three cultures were used, two standard baking yeasts, strains U and R (*S. cerevisiae*), and a hybrid (*S. cerevisiae* by *S. globosus*). Strain U stored both fat and carbohydrate relatively uniformly, as indicated by microscopic examination. Strain R stored carbohydrate well, but the accumulations of fat were irregular and the fat-containing culture was discarded. The hybrid stored fat in large clusters of extremely tiny granules approaching the limits of visibility. The cells appeared to be crowded with stored materials,

TABLE II  
*Chemical Analyses of Cells Containing Visible Deposits of Fat and  
Glycogen Compared to Normal Cells*

Culture	Visible Deposit	Protein	Fat	Ash	Carbo- hydrates
U	None	61.3	4.83	8.48	25.4
	Fat	25.0	31.25	5.22	38.5
	Glycogen	31.9	16.96	5.33	45.7
C × GII	None	54.4	.17	9.29	36.1
	Fat	39.9	6.16	7.09	47.3
R	None	53.8	7.47	9.15	29.5
	Glycogen	31.9	5.24	5.22	57.7

but chemical analysis (other extraction) revealed that only 6.16% of fat was present. The hybrid stored carbohydrate poorly. Nitrogen analyses were converted to protein by multiplying by the factor 6.25 (Table II). The sample was ashed, and the difference was calculated to be carbohydrate.

Manometric analyses were made on cells containing no visible deposits and compared with those made on cells containing deposits of glycogen and fat. The results are shown in Table III. When a constant rate of oxygen consumption or CO<sub>2</sub> evolution was reached, the curve was extrapolated to the x-axis to determine the lag period. In the absence of corn-steep water, maximal activity was attained in a short time, thirty minutes generally; however, in the presence of corn-steep water, an hour or more was usually required to attain a

TABLE III

*The Effect of Stored Reserves on Respiratory and Fermentative Activity of Cells in Different Nutrient Solutions*

Visible Deposit	Nutrient Added to Warburg vessel	Q <sub>o</sub>	Lag in Min	Q <sub>o</sub> <sup>O</sup>	Fig	Q <sub>o</sub> <sup>N</sup>	Lact	Q <sub>o</sub> (N)	Q <sub>o</sub> <sup>O</sup> (N)	Q <sub>o</sub> <sup>N</sup> (N)
Culture U										
None	Glu.	10	0	160	0	276	0	102	1631	2420
None	Glu	137	5	348	0	284	0	1400	3550	2900
Gly.	None	88	5	88	5	0		1725	1725	0
Gly.	CSW	160	60	133	55	0		3140	2610	0
Gly.	Bi. + Pan	0		0		0		0	0	0
Fat	None	12	10	32	15	0		1050	800	0
Fat	CSW	85	50	78	42	0		2120	1950	0
Fat	Bi. + Pan	33	25	30	25	0		825	750	0
Gly.	Glu.	0		36	0			0	706	
Gly.	Glu. + CSW	121	10	1240	100			2430	24300	
Gly.	Glu. + Bi. + Pan.	0		21	0			0	412	
Fat	Glu.	31	5	13	0			775	1075	
Fat	Glu + CSW	77	60	530	130			1925	13250	
Fat	Glu + Bi + Pan	32	0	28	0			800	700	
Culture C × GII										
None	Glu	60	0	421	5	1308	0	690	4840	3540
None	Glu	71	0	377	10	241	0	850	1330	2770
Fat	None	26	60	11	60	1	0	413	175	61
Fat	CSW	40	80	30	30	8	0	635	476	127
Fat	Glu	16	70	35	0	34	0	254	555	540
Fat	Glu + CSW	17	0	97	15	328	8	270	1510	5200
Culture R										
None	Glu.	83	0	370	5	278	0	964	1300	3230
None	Glu	109	5	432	5	299	0	1266	5010	3170
Gly.	None	0		0				0	0	
Gly.	CSW	205	97	191	115			4020	3740	
Gly.	Glu.	38	43	174	127			745	3410	
Gly.	Glu. + CSW	259	183	938	220			5080	18400	

Abbreviations:

Bi — Biotin  
CSW — Corn-Steep Water  
Glu. — Glucose

Gly Glycogen  
Pan. Pantothenic Acid

constant maximal activity although consumption of  $O_2$  and evolution of  $CO_2$  began at a low rate almost as soon as readings were taken. When a full nutrient was used, growth of the yeast occurred so that the simple fermentative or respiratory activity was not being recorded, and the  $Q$  values have no precise meaning, for the amount of yeast in the vessels increased. However, the data are recorded, for, when they are considered along with the long lag period, they indicate that the inhibiting effects of the stored reserves have been overcome and normal growth has started.

The manometric tests of the controls (tests on cells without reserves) were made with 4% glucose in the Warburg vessels and the average values of  $Q_{O_2}:Q_{CO_2}^0$  were 79:351, which is normal for cells free of reserves, i.e., for standard bakers' yeast as it is ordinarily prepared. Comparable tests on the cells with either stored fat or carbohydrate (also in 4% glucose) gave average values of about 21:72, indicating the reserves inhibited respiration. This is further confirmed by a calculation of the activity based on the total nitrogen rather than on the total dry weight.

These average values, as well as the data in Table III, must be considered as indicating trends rather than precise values for it was not possible to run all the tests at the same time and some changes of the yeasts may have occurred while they were stored in the cold room. Furthermore, although a manufactured bakers' yeast can be produced from day to day, year in and year out, with a highly predictable range of  $Q$  values, when yeasts are grown in small batches in a laboratory they are usually quite variable. The present experiments indicate, of course, that the principal factor in this variability is the reserves. However, it is quite difficult to get exactly the same amount of reserves in two cultures made on different days. Although the general tendency of the reserves to reduce respiratory and fermentative activity is quite clear, it is quite another thing to devise experiments in which precisely the same amount of reduction of activity will be attained. Minor factors, such as the amount of inoculum, can change this very greatly. For these reasons the data in Table III cannot be analyzed too rigidly but must be merely used to indicate trends.

Table III reveals that the R.Q. of cells with stored carbohydrate was usually about unity when tested in the absence of substrate. This observation confirms the findings of Stier and Stannard (1935a, b;



FIG. 1

Yeast Cells Containing Glycogen After Two Hours in a Warburg Vessel

1937) and Spiegelman and Nozawa (1945), who reported that intact yeast cells oxidize, rather than ferment, their carbohydrate reserves.

Spiegelman and Nozawa studied six strains of yeast and found that the average  $Q_{O_2}:Q_{CO_2}^O:Q_{CO_2}^N$  in 4% glucose in a Warburg vessel was 78:216:288. In the absence of glucose the values were respectively 27:27:0. They grew their cultures for forty-eight hours in 8% glucose broth without shaking and under these circumstances visible glycogen is usually deposited in the cells. When cells without visible reserves are tested manometrically in the absence of substrate, the values are 0:0:0.

The data in Table III show that an accumulation of fat reserves may reduce the  $Q_{CO_2}^N$  values in the presence of glucose from around 250 to about 30 and we have made observations on other cultures with stored glycogen which show that the ability to ferment external substrate may be reduced to zero.

However, visible glycogen does not always reduce the  $Q_{CO_2}^N$  value of zero. When glycogen is stored in the cell either in the form of one large granule or in the form of numerous small, well-dispersed granules, the  $Q_{CO_2}^N$  value in the presence of external substrate may be over 200. Fig. 1 shows the accumulation of glycogen in cells removed from a Warburg vessel which has been flushed with nitrogen and which contained 4% glucose buffer solution. The cells are stained with iodine and the loosely-packed dark material is glycogen. These cells are fermenting actively, showing clearly that loosely-packed glycogen did not inhibit anaerobic fermentation of external substrate. This amount of glycogen was deposited in less than two hours.

Cells containing stored fats oxidize the fat reserves with an R.Q. of less than 1.

Biotin (2 $\gamma$ /l) and pantothenic acid (200 $\gamma$ /l) were added to some of the Warburg vessels as indicated. Apparently biotin was unable to overcome the inhibitory effects of the accumulated reserves.

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# The Nutritional Role of Acetate for Lactic Acid Bacteria

## I. The Response to Substances Related to Acetate

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### INTRODUCTION

The stimulatory effect of sodium acetate on growth of lactic acid bacteria was first demonstrated by Snell *et al.* (1). These investigators suggested that, in addition to its role as buffer, sodium acetate may have a specific function concerned with the initiation of growth (2).<sup>1</sup> Since these early observations acetate has been included routinely in media used for microbiological assays for vitamins and amino acids employing lactic acid bacteria.

Experiments described below indicate that acetate may serve a dual purpose in media, (a) that of buffer, to prevent the lactic acid formed during growth from lowering the pH of the medium to toxic levels, and (b) an unknown function, concerned with the production of early and luxuriant growth. The purpose of this investigation was to elucidate this unknown function.

As one approach to the problem, substances reportedly formed from acetate or capable of degradation to acetate were tested for their ability to duplicate the growth effect of acetate for various representative organisms of the lactic acid group. The results of these assays and others in which many related compounds were tested provide evidence indicating a probable role for acetate in the synthesis of lipoidal materials by lactic acid bacteria.

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<sup>1</sup> Gilbert (3) recently demonstrated the accelerating effect of sodium acetate on growth of pneumococci and concluded that this effect was not due to a buffering action.

## EXPERIMENTAL

*Test Organisms.* The species of lactic acid bacteria used in this investigation were: *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5, *Lactobacillus acidophilus* and, less frequently, *Streptococcus lactis* 125 and *Lactobacillus pentosus* 121-2.

*Testing Procedure.* An acetate-free, phosphate-buffered medium was used for all tests. The composition of the double-strength medium is given in Table I.

TABLE I  
*Acetate-Free Basal Medium*

Hydrolyzed casein <sup>1</sup>	1.0 g.
Potassium dihydrogen phosphate	0.5 g.
<i>l</i> -Tryptophane	5.0 mg.
<i>l</i> -Cystine hydrochloride	20.0 mg.
<i>l</i> -Asparagine	20.0 mg.
Salts A <sup>†</sup>	1.0 cc.
Salts B <sup>‡</sup>	1.0 cc.
Adenine sulfate	2.0 mg.
Guanine hydrochloride	2.0 mg.
Uracil	2.0 mg.
Thiamine hydrochloride	20.0 $\gamma$
Calcium pantothenate	20.0 $\gamma$
Riboflavin	20.0 $\gamma$
Nicotinic acid	20.0 $\gamma$
<i>p</i> -Aminobenzoic acid	20.0 $\gamma$
Biotin	0.16 $\gamma$
Inositol	10.0 $\gamma$
Pyridoxine hydrochloride <sup>§</sup>	200 $\gamma$
Folic Acid concentrate <sup>  </sup>	20.0 mg. u.
Glucose	2.0 g.
Adjust to pH 6.5	
Distilled water to	100 cc.

\* Vitamin-free Labco Casein was hydrolyzed with hydrochloric acid according to the procedure described in The University of Texas Publication No. 1137. The hydrolyzate was preserved under toluene and treated again with charcoal (10% Dureco G-60) at pH 3.0 before incorporation into the medium.

† Salts A contained 25 g. each of  $KH_2PO_4$  and  $K_2HPO_4$  per 250 cc. of solution.

‡ Salts B contained 10 g. of  $MgSO_4 \cdot 7H_2O$  and 0.5 g. each of  $FeSO_4 \cdot 7H_2O$ ,  $MnSO_4 \cdot 4H_2O$ , and  $NaCl$  per 250 cc. of solution.

§ For *L. arabinosus*, the replacement of pyridoxine with 0.1  $\gamma$  pyridoxamine dihydrochloride per 10 cc. of culture medium resulted in a better growth response. A solution of pyridoxamine dihydrochloride (100  $\gamma$  per cc.) stored in the refrigerator was stable for several weeks.

|| "Potency 3100" -- Mitchell, Snell, and Williams (4).

It was found convenient to combine the purines and pyrimidines into one solution and the vitamins into another. These solutions were stored in the refrigerator and the vitamin supplement renewed each month. The remaining components of the medium, with the exception of glucose, which was added just prior to pipetting the medium into assay tubes, were combined into another solution. The latter solution was preserved by steaming for 10 minutes after withdrawal of aliquots.

The testing procedure is essentially that described by Snell and Wright for nicotinic acid (5). The standard is made up of a series of tubes containing sodium acetate in the following ranges for the various organisms: 1.0 to 10 mg. for *L. casei* and *L. acidophilus*, 0.1 to 1.0 mg. for *L. arabinosus*, *S. lactis* and *L. pentosus*. Neutralized solutions of the substances to be tested were similarly added to the medium in graded amounts and the growth promoting activity, if any, compared to that of acetate.

When the materials tested were water-insoluble, appropriate solutions in ethanol were added to the double-strength basal medium, which was then diluted to the proper volume (10 cc.) with water. This procedure materially lessened the tendency of the water-insoluble materials to separate from the test medium. In only a few cases was visible turbidity of the medium produced by addition of such samples; in these cases appropriate uninoculated controls were carried to permit differentiation of turbidity due to sample and that due to growth of the test organism. In doubtful cases, extent of growth was determined by plate counts, or acidity measurements were made in place of turbidity measurements. In all cases in which alcohol was added to the test medium, it was determined by separate tests that the amounts used were without effect on growth of the test organism. In general, additions of alcohol greater than 0.2 cc. per 10 cc. of medium proved toxic to the test organisms and were not employed.

When heat-labile, or extremely volatile compounds, or substances which were likely to react with components of the medium during heat sterilization were tested, these were added aseptically to the appropriately diluted medium after the test had been autoclaved and cooled. Solutions of water-soluble compounds were sterilized by filtration.

When *L. arabinosus* was used as the test organism, one drop of a barely visible suspension was used to inoculate each assay tube. For each of the remaining test organisms, the density of the inoculum employed was roughly ten times that used with *L. arabinosus*. *L. casei* was incubated at 37°C. for 15 to 24 hours, *L. acidophilus* at the same temperature for 24 hours. *L. arabinosus* and *S. lactis* were incubated at 30-33°C. for a period of 15 to 24 hours.

*Measurements of Growth Response.* Growth response was followed turbidimetrically or acidimetrically and there was a close correspondence between results obtained by the two methods of measurement. As the quantity of acid produced by these organisms during an incubation period of 24 hours is not appreciable, growth response was for the most part measured turbidimetrically. The instrument used was set to read zero with distilled water and 100 when no light was transmitted.

A curve relating galvanometer readings to dry weight of cells was constructed by making serial dilutions of a cell suspension of known weight per unit volume (obtained by determining dry weight of an aliquot). The dry weight of cells corresponding

to a given turbidity produced in response to a test substance could then be obtained by referring to the curve.

*Factors Affecting Growth Response.* The time in which maximum growth response to stimulatory materials was obtained with any given organism varied over a rather wide range and was affected by the size of the inoculum, the nature of the test substance, and by other factors yet unknown.

The response of *L. arabinosus* appears to be most susceptible to changes in size of inoculum. Best results were obtained with this organism when assays were carried out in short tubes of uniform dimensions (22 × 100 mm.). Use of the short tubes made it easier to introduce the drop of inoculum directly into the medium, thus assuring a uniform distribution of inoculum among the culture tubes.

In general, the organisms responded more slowly to water-insoluble substances, e.g., cholesterol, than to water-soluble ones such as acetate.

## RESULTS

*The Response to Acetate.* The response to sodium acetate of each of the organisms used in this investigation is illustrated by Figs. 1 and 2.

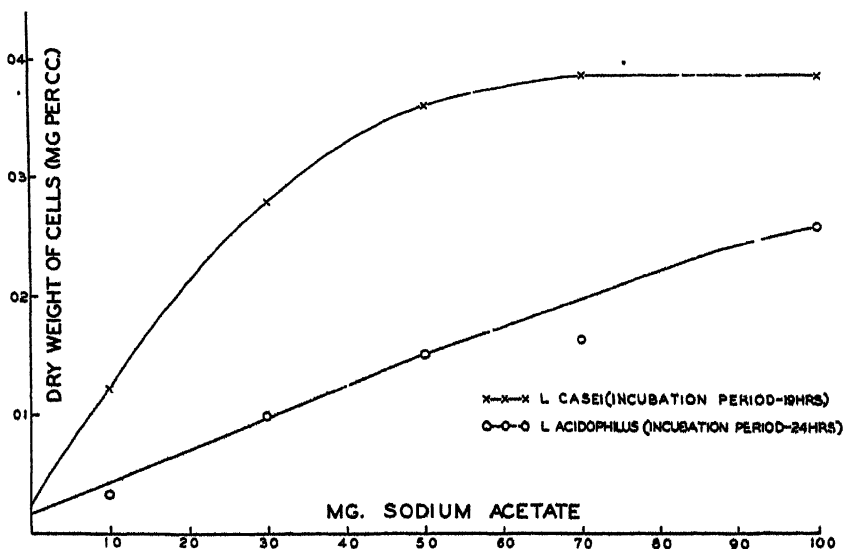


FIG. 1

Response of *L. casei* and *L. acidophilus* to Sodium Acetate in Media Buffered with Phosphate

The amount of acetate necessary to stimulate early growth of these organisms is much less than the quantity of acetate usually added to

media for the purpose of serving as buffer (*i.e.*, from 0.6 to 2.0%). The data of Table II show that the stimulatory effect of sodium acetate was not duplicated by equimolar quantities of sodium propionate, potassium citrate or potassium dihydrogen phosphate. These data

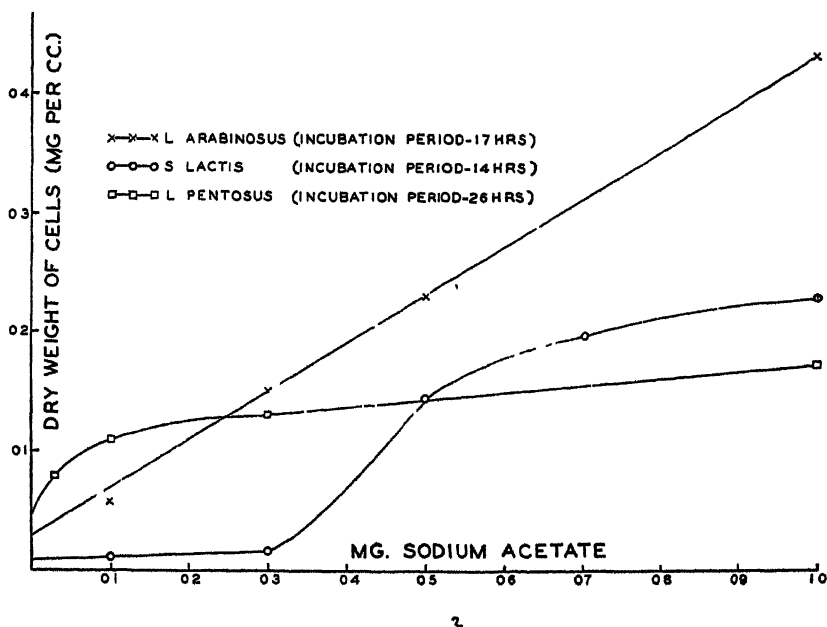


FIG. 2

Response of *L. arabinosus*, *S. lactis* and *L. pentosus* to Sodium Acetate in Media Buffered with Phosphate

demonstrate that acetate serves some other function(s) beside that of buffer for these organisms. The response to acetate was improved by the presence in the medium of either phosphate or citrate as indicated by Table II, but was not improved appreciably by the presence of propionate.

*Growth in the Absence of Acetate.* The effect of acetate is greatly to stimulate rate of growth and to permit its more ready initiation. Under the conditions used, some growth often appeared in the control tubes which contained no added acetate; if the incubation period was greatly prolonged, fairly heavy growth eventually occurred without

TABLE II

*The Comparative Effect of Sodium Acetate and Other Buffers on Growth of Lactic Acid Bacteria*

Additions* to 10 cc of culture medium	Dry weight of cells obtained (mg /10 cc)		
	<i>L. casei</i> †	<i>L. acidophilus</i> ‡	<i>L. arabinosus</i> §
a. None	0.1	0.12	0.1
b. Acetate (1.0 mg.)	—	—	4.1
c. Acetate (10 mg.)	2.4	1.22	—
d. Phosphate (1.4 mg.)	—	—	0.13
e. Phosphate (14 mg.)	0.22	0.3	—
f. Phosphate (25 mg.)	0.3	0.15	0.23
g. Citrate (3.2 mg.)	—	—	0.18
h. Citrate (25 mg.)	0.13	0.31	0.64
i. Citrate (32.4 mg.)	0.2	0.31	—
j. Acetate (1.0 mg.) + Phosphate (25 mg.)	1.6	0.62	5.8
k. Acetate (5.0 mg.) + Phosphate (25 mg.)	—	3.75	—
l. Acetate (10 mg.) + Phosphate (25 mg.)	6.4	—	—
m. Acetate (1.0 mg.) + Citrate (25 mg.)	1.0	1.95	6.85
n. Acetate (5.0 mg.) + Citrate (25 mg.)	—	4.5	—
o. Acetate (10 mg.) + Citrate (25 mg.)	4.4	—	—
p. Propionate (1.0 mg.)	—	—	0.14
q. Propionate (10 mg.)	0.3	0.35	—

\* Acetate refers to sodium acetate, phosphate to potassium dihydrogen phosphate, citrate to potassium citrate monohydrate and propionate to sodium propionate.

† Incubated 17 hours.

‡ Incubated 24 hours.

§ Incubated 24 hours.

added acetate. Such slow growth might occur without the aid of acetate, or it might result from the normal fermentative production of small amounts of acetate from glucose or from the amino acids present in the medium. Enrichment of the medium by addition of extra amino acids stimulated growth of *L. arabinosus* and *S. lactis* slightly, but the effect could not be attributed to any single amino acid and was much less marked than the effect produced by equal amounts of sodium acetate.

*The Response of Lactic Acid Bacteria to a Miscellaneous Group of Metabolites and Precursors of Acetate.* In this group the following compounds were tested for their ability to replace acetate in stimulating

growth of the lactic acid bacteria: acetone, ethyl alcohol, oxalic acid, glycollic acid, acetoacetic acid,<sup>2</sup> and  $\beta$ -hydroxybutyric acid.

Acetoacetic acid was from 0.1 to 4.4 times (by weight)<sup>3</sup> as active as sodium acetate in promoting growth of *L. arabinosus* on the acetate-free medium. It also stimulated growth of *S. lactis* (0.25 acetate equivalents per mg.), but was without activity for *L. casei* and *L. acidophilus*. The instability of the keto acid probably explains the variability in response of *L. arabinosus* to it. Evidently, the different organisms tested differ considerably in their ability to utilize this substance. All of the other compounds tested were inactive, with the possible exception of acetone, which showed a very slight growth-promoting effect with *L. arabinosus* only.

*The Response to Various Products Involved in Carbohydrate Metabolism.* Succinic, malic,  $\alpha$ -ketoglutaric,<sup>4</sup> glyceric, phosphoglyceric<sup>4</sup> and inosinic<sup>4</sup> acids, creatine and creatinine were inactive in stimulating early growth of the lactic acid bacteria when tested on the acetate-free medium. Fumaric, citric, *l*-aspartic and *l*-glutamic acids were slightly stimulatory to *L. arabinosus* only. However, the stimulation was much less than that produced by an equivalent weight of sodium acetate.

Oxaloacetic acid<sup>4</sup> was active in replacing acetate for *L. arabinosus*. The activity of this substance varied from 0.16 to 3.4 acetate equivalents per mg. As in the case of acetoacetic acid, the instability of the compound may account for the variability in response. Since oxaloacetic acid is readily decarboxylated by microorganisms, the known decarboxylation products—acetoin (7), acetaldehyde (8), pyruvic acid (9, 10)—were tested to determine whether or not oxaloacetic acid might be utilized by *L. arabinosus* via this reaction. The assay value for acetoin was 0.28 acetate equivalents per mg. Sodium pyruvate<sup>4</sup> <sup>5</sup>

<sup>2</sup> Obtained by hydrolysis of acetoacetic ester according to the method used by Smedley-MacLean and Hoffert (6). An 8-hour instead of a 24-hour hydrolysis period was used.

<sup>3</sup> Subsequently, the activity of any given compound in promoting growth will be expressed in terms of acetate equivalents. Thus, in the above example, acetoacetic acid varied in activity from 0.1 to 4.4 acetate equivalents per mg.; that is, one mg. of acetoacetic acid is equivalent in growth-promoting activity to 0.1–4.4 mg. of sodium acetate.

<sup>4</sup> Kindly supplied by Dr. F. Schlenk, M. D. Anderson Hospital for Cancer Research, Houston, Tex.

<sup>5</sup> Sodium pyruvate was inactive for all other organisms tested.



and acetaldehyde<sup>6</sup> possessed only slight and variable activity of *L. arabinosus*, acetaldehyde being toxic at a concentration of 0.1 mg./cc. of culture medium and above. As pyruvate is such a universal intermediate in bacterial metabolism and gives rise to acetic acid in other bacteria by so many different pathways (11) its inactivity as a substitute for acetate is rather surprising. Oxaloacetic acid might be hydrolyzed by the organism to yield acetic and oxalic acids, and the occurrence of this reaction may partially explain the activity of this compound in replacing acetate.

It is evident from the results of these tests that most of the recognized intermediates of carbohydrate metabolism fail to replace acetate in its growth-stimulating role for lactic acid bacteria.

*The Response to Fatty Acids.* The testing of fatty acids promised to be informative for two reasons. The synthesis of higher fatty acids from the lower members of the series has often been postulated, and in the case of the rat, the synthesis of fatty acids from acetic acid (12) has been proven. Also, acetic acid may arise from the dissimilation of fatty acids. The response of *L. arabinosus* to saturated and unsaturated fatty acids is given in Table III.

The lower members of the fatty acid series (formic, propionic, butyric, valeric, isovaleric and caproic acids) did not stimulate growth of these bacteria during short periods of incubation. Beginning with caprylic acid, the saturated fatty acids were active for *L. arabinosus*, myristic acid displaying the highest activity. Palmitic acid was less active, while stearic acid was inactive.<sup>7</sup> The unsaturated acids were active for *L. arabinosus* and their activity is of such magnitude that they are indicated as being intermediates or end-products of acetate metabolism. These results can thus be considered confirmatory to those of Rittenberg and Block (12) obtained with rats and mice.

In contrast to the response obtained with *L. arabinosus*, all of the fatty acids tested were inactive for *L. casei*. These results demonstrate a fundamental dissimilarity in behavior between the two organisms. *L. acidophilus* occupies a position intermediate between *L. arabinosus* and *L. casei* with respect to its response to the fatty acids; the unsat-

<sup>6</sup> For *S. lactis*, acetaldehyde gave an average assay value of 1.9 acetate equivalents per mg. On the molar basis then, acetaldehyde and sodium acetate are equally active for this organism.

<sup>7</sup> Stearic acid is precipitated by the inorganic salts of the medium; satisfactory determination of its activity was therefore difficult.

urated acids display some activity for this organism, but the saturated acids are inactive.

Triacetin, glyceryl monooleate and lecithin were also tested and the results obtained with *L. arabinosus* are appended to Table III. Gly-

TABLE III

*The Response of L. arabinosus to Fatty Acids in an Acetate-Free Medium*

Substance tested (number of times given in parentheses)	Range tested <sup>1</sup> (mg.)	Activity (acetate equivalents per mg.)	
		(Range)	(Average)
Caprylic acid† (3)	0.1 -1.0	0.13- 3.8	1.4
Capric acid† (3)	0.01 -1.0	3.1 - 6.9	5.2
Lauric acid (4)	0.001-0.3 (0.01 -0.1)	1.0 - 8.3	3.3
Myristic acid (4)	0.001-0.3 (0.03 -0.05)	5.0 - 18	12
Palmitic acid (3)	0.001-0.5 (0.03 -0.3)	0.32- 4.5	2.1
Stearic acid (2)	0.001-0.5	inactive	
Oleic acid (12)	0.001-1.0 (0.001-0.01)	43 -230	80
Linoleic acid (3)	0.001-0.5 (0.001-0.05)	26 - 67	42
Linolenic acid (5)	0.001-0.5 (0.001-0.05)	18 - 51	31
Sodium ricinoleate (3)	0.005-0.1 (0.01 -0.1)	6.8 - 8.5	7.7
Glyceryl monooleate (1)	0.01 -0.1	24	(24)
Triacetin (1)	0.01 -1.0	inactive	
Lecithin (4)	0.005-0.1	5.1 - 34	19

\* When "active range" does not correspond to "range tested," it is given in parentheses below the latter.

† The product (Armour and Company) contains other fatty acids to the extent of 10%.

ceryl monooleate showed only one-third the activity to be expected from its content of oleic acid. It was inactive for *L. casei*. Triacetin was inactive for *L. casei* as well as for *L. arabinosus*. Lecithin was active for all three organisms tested (0.1 and 7.1 acetate equivalents per mg. for *L. casei* and *L. acidophilus*, respectively). If the phospholipid is hydrolyzed by these organisms the activity must be as-

cribed to the fatty acid component, since both glycerol and choline were inactive for these bacteria.

Acetic acid is a possible end-product of fatty acid oxidation by any of the theories now extant, and such degradation to acetic acid may partially explain the activity of these substances for lactic acid bacteria. However, complete degradation of some of these substances (e.g., oleic acid) to acetic acid would be insufficient to account for their high activity. This suggests that they may be normally synthesized from acetate and, hence, are capable of replacing acetate under the conditions employed, serving as growth factors directly or as intermediates in the further synthesis of essential materials. Oleic acid has been previously reported to serve as a growth factor for *Corynebacterium diphtheriae* (13) and for *Clostridium tetani* (14).

TABLE IV  
*Comparative Response of L. arabinosus to Acetate and Sterols as Indicated by Plate Counts*

Addition to 10 cc. of culture medium	Galvanometer (turbidity) readings (incubated 20 hrs.)	Average number of cells per cc. (from plate counts)	Activity (acetate equivalents per mg.)
None	44.5	$4.7 \times 10^7$	
Sodium acetate (0.1 mg.)	68.5	$1.5 \times 10^8$	
Sodium acetate (0.3 mg.)	76.0	$4.5 \times 10^8$	
Cholesterol (0.1 mg.)	76.5	$3.2 \times 10^8$	2.0
Ergosterol (0.1 mg.)	80.0	$5.0 \times 10^8$	3.0

*The Response to Sterols and Related Compounds.* The unsaponifiable fractions of most bacterial cells, unlike those of other living cells, do not show the characteristic reactions of sterols (15, 16). The only species of bacteria which has been reported to contain significant quantities of digitonin-precipitable sterols is *Azotobacter chroococcum* (17). However, the results of experiments of Sonderhoff and Thomas with yeast (18) and Block and Rittenberg with rats (19) concerning the utilization of acetate for sterol synthesis, and also the finding that several parasitic flagellates require sterols as growth factors (20, 21) stimulated the investigation of this group of compounds in relation to lactic acid bacteria.

When cholesterol was introduced into the acetate-free culture medium, growth of each species of lactic acid bacteria tested was stimulated. For the preliminary experiments growth response was

measured by means of bacterial plate counts. Data presented in Table IV definitely demonstrate a stimulation in growth of *L. arabinosus* by cholesterol and ergosterol. Similar results were obtained with *L. casei*; the addition of 5 mg. of cholesterol to a 10 cc. culture tube produced a 10-fold increase in cell population over the control tubes.

The results of more detailed testing of sterols and related compounds are recorded in Table V.

The lack of correspondence between growth curves obtained with sterols and those obtained with sodium acetate made direct comparisons of activity with sodium acetate difficult and sometimes rather unsatisfactory. In such cases, values for activity were calculated in terms of cholesterol, then converted to acetate equivalents by multiplying by the appropriate factor (3.8 in the case of *L. arabinosus*, which represents the average activity of cholesterol in terms of acetate for this organism).

Of the organisms employed, *L. arabinosus* was most responsive to the sterols and related compounds, *L. acidophilus* less so and *L. casei* responded to only a few. The activity of the sterols in replacing acetate suggests that these compounds are in some way connected with acetate metabolism. They may be utilized directly or converted into other essential substances. The inactivity of most of these compounds for *L. casei* may mean that either they are not assimilated from the medium or not utilized. It is possible that differences in permeability of cells of *L. casei* and *L. arabinosus* may account for differences in behavior of these two organisms toward the steroids tested.

The difficulty in obtaining uniform dispersions of these water-insoluble materials in the culture medium probably contributes greatly to the variability observed in the activity of a single compound when tested repeatedly.

For *L. arabinosus* and *L. acidophilus* members of the three classes of sterols, namely, the zoosterols, phytosterols and mycosterols, were active. Also, the sterol ketones: cholestanone, cholestenone and coprostanone, and the derived sterols:  $\beta$ -cholestanol, 7-dehydrocholesterol, epidihydrocholesterol and neoergosterol were active for *L. arabinosus* (and certain of these for *L. acidophilus* and *L. casei*). The irradiation products of ergosterol and 7-dehydrocholesterol, calciferol and vitamin D<sub>3</sub>, respectively, were more active than the original sterols for *L. arabinosus*. Calciferol was many times more active than ergos-

TABLE V  
The Response of Lactic Acid Bacteria to Sterols and Related Compounds

Substance tested	Activity (acetate equivalents/mg.) for						Remarks
	<i>L. casei</i>		<i>L. arabinosus</i>		<i>L. acidophilus</i>		
	(Range)	(Av.)	(Range)	(Av.)	(Range)	(Av.)	
Abietic acid	inactive		1.4, 4.0	2.7	14.5, 19	17	Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.
Allopregnandione-3,20†	inactive		(0.23-0.33)	1.1*	—	—	Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.
Allopregnanetriol-3(α),16,20†	1.7	1.7	(0.32-1.2)	4.2*	—	—	
Bile salts	inactive		0.83, 3.0	1.9	3.1, 2.0	2.5	Maximum dose tested ( <i>L. casei</i> ): 10 mg./10 cc.
Calciferol‡	inactive		2.7-10.9	6.4	103, 140	122	Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.; added after sterilization of medium.
Cholestanol†	inactive		1.9, 1.2	1.6	—	—	Maximum dose tested ( <i>L. casei</i> ): 0.2 mg./10 cc.
β-Cholestanol§	1.7	1.7	0.29	0.29	—	—	
Cholestanone	—		0.48	0.48	—	—	
Cholestanone‡	0.7, 1.5	1.1	0.83, 1.2	1.0	—	—	

Cholic acid, digitan, phenanthrene, sitosterol and sodium desoxycholate were inactive for all organisms tested.

\* The values in parenthesis are expressed in terms of cholesterol equivalents per mg.; the value in the average column was obtained by multiplying cholesterol equivalents per mg. by the average activity of cholesterol in terms of acetate for the particular organism concerned.

† Kindly supplied by Dr. E. Rohrmann, The Lilly Research Laboratories.

‡ Kindly supplied by Dr. A. L. Bacharach, Glaxo Laboratories.

§ Kindly supplied by Dr. O. Rosenheim, National Institute for Medical Research, Hamstead, London.

|| Kindly supplied by Dr. H. M. Crooks, Jr., Parke, Davis and Company.

TABLE V—Continued

Substance tested	Activity (acetate equivalents/mg.) for					Remarks
	<i>L. casei</i>		<i>L. arabinosus</i>		<i>L. acidophilus</i>	
	(Range)	(Av.)	(Range)	(Av.)	(Range) (Av.)	
Cholesterol	0.75-3.7	1.5	1.4-10	3.8	1.0-29   16	For <i>S. Lactis</i> the activity of cholesterol averaged 73 acetate equivalents/mg., for <i>L. pen-tosus</i> , 3.0 acetate equivalents/mg.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc. Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.  Maximum dose tested ( <i>L. casei</i> ): 0.4 mg./10 cc.; added after sterilization of medium
Coprostanone†	1.3, 1.5	1.4	5.4, 10.3	7.9	—	
7-Dehydrocholesterol†	inactive	—	5.5	5.5	—	
Digitonin	inactive	—	(0.22, 0.26)	0.9*	—	
Epidihydrocholesterol	2.0	2.0	(0.64, 0.51)	2.2*	—	
Equilenin†	inactive	—	0.55-2.4	1.8	—	
Ergosterol	inactive	—	1.2-4.0	2.6	—	
Estrone†	inactive	—	0.7-0.93	0.85	inactive	
Isoandrosterone†	inactive	—	(0.21, 0.33)	1.0*	3.4, 4.7   4.1	
Neogosterol†	—	—	2.3-7.1	4.5	16.9, 24   20.5	
Pregnandione-3, 20†	inactive	—	0.69-0.88	0.79	—	
Progesterone	inactive	—	1.2-3.3	2.2	—	
Saponin (Eastman Kodak)	0.3, 0.5	0.4	0.1-0.46	0.22	—	
Sodium glycocholate	inactive	—	17	17	—	
Stigmasterol†	—	—	0.81-1.3	1.1	12.5, 8.1   10.3	
Strophanthin	—	—	0.1	0.1	—	
Uranediol-3(β), 11†	1.5	1.5	1.1-4.1	2.4	—	
Vitamin D <sub>2</sub> †	inactive	—	6.4	6.4	—	

For *S. Lactis* the activity of cholesterol averaged 73 acetate equivalents/mg., for *L. pentosus*, 3.0 acetate equivalents/mg.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*, *L. acidophilus*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc.

Maximum dose tested (*L. casei*): 10 mg./10 cc.

Maximum dose tested (*L. casei*): 0.4 mg./10 cc., added after sterilization of medium

terol for *L. acidophilus*. Calciferol and vitamin D<sub>3</sub> were inactive for *L. casei*.

The bile acids are closely related in structure to the sterols and evidence for the biological conversion of sterol to bile acid by the dog has been presented recently (22). The presence of bile acids in bacteria has not been demonstrated, nor has the utilization of these compounds by bacteria been reported. However, a mixture of bile salts was active for *L. arabinosus* and *L. acidophilus* and showed a trace of activity for *L. casei*. The activity of this product was probably due to the presence of glycocholic acid, which showed high activity for *L. arabinosus*. Cholic and desoxycholic acids were inactive for all of the organisms tested.

Reference to Table V shows that some of the sex hormones, the saponins and one of the cardiac glycosides also possessed some activity for one or more of the organisms employed. All of these compounds are closely related in structure to the sterols. The activity of the saponins must be ascribed to the aglycone portion since ether extraction of an acid hydrolyzate of saponin removed the active material almost quantitatively. The purified saponin, digitonin, possessed slight activity for *L. arabinosus* but was inactive for *L. casei*.

The biogenesis of steroids has been a subject of much speculation. Much evidence (18, 19) has shown that acetate functions as such a precursor for yeast and rats. The demonstration that steroids partially duplicate the growth-promoting effect of sodium acetate for lactic acid bacteria thus assumes added significance. Certain other postulated precursors, such as acetaldehyde and fatty acids, have also been shown to possess similar activity for these organisms, although the activity of acetaldehyde was questionable.

Evidence for the presence of steroid material in lactic acid bacteria grown on a sterol-free medium is furnished by the experiment described below. The unsaponifiable fractions of the bacterial cells (of *L. casei*, *L. arabinosus* and *L. acidophilus*) were isolated by the technique of Hilditch (23) and the Liebermann-Burchard color test was applied to each fraction. A positive reaction was obtained in every case. Biological tests of this nonsaponifiable material showed it to be very effective in replacing acetate for *L. arabinosus* and *L. acidophilus* (Table VI). Indeed, it was more active for *L. arabinosus* than any of the pure compounds tested.

TABLE VI

*The Response of the Lactic Acid Bacteria to Unsaponifiable Fractions of Bacterial Cells*

Unsaponifi- able fraction of	Quantity	Activity		
	per cent by weight of dried cells	Acetate equivalents		
		mg. of material extracted		
		<i>L. arabinosus</i>	<i>L. casei</i>	<i>L. acidophilus</i>
<i>L. arabinosus</i>	2.1	124	0	125
<i>L. casei</i>	10	27	1.0	—
<i>L. acidophilus</i>	2.9	42	0	44

*The Response to Terpenes, Carotenoid Pigments and Related Compounds.* The recognized structural relationships between the sterols, terpenes and carotenoids suggested that representatives of the latter two classes of compounds also be tested for their activity in promoting growth of lactic acid bacteria in the absence of acetate. Certain terpenes (Table VII) possessed slight activity for *L. arabinosus*, the

TABLE VII

*The Response of L. arabinosus to Terpenes and Related Compounds*

Substance tested	Activity (acetate equivalents/mg.)
<i>dl</i> -Camphonanic acid	trace*
Camphor	0.3, 0.11 (av., 0.21)
Camphoric acid	inactive
Cineole	inactive
Citral	0.12
Cymene	0.26, 0.17 (av., 0.22)
Dipentene	0.89
Geraniol	0.53, 0.23 (av., 0.38)
Ionone	0.35–5.6 (av., 2.6)
Isoprene	0.1–0.6 (av., 0.35)
Phytol	0.6, 0.6 (av., 0.6)
Terpineol	0.11
2,2,6-Trimethyl- cyclohexane-1- carboxylic acid	trace

\* Compounds which are described as possessing a trace of activity stimulate growth above the blanks but do not support increasing growth of the organism with increasing concentration of the test substance.

organism for which the sterols were most active. They were inactive for *L. casei*. Certain carotenoid pigments were likewise active in replacing acetate for *L. arabinosus* (Table VIII). In general, the hydro-



TABLE VIII  
*The Response of L. arabinosus to Carotenoid Pigments*

Pigment	Source	Activity (acetate equivalents mg.)
Carotene	Commercial product (85% $\beta$ ; 15% $\alpha$ )	0.69-25
$\beta$ -Carotene	Chromatographic separation of commercial carotene	9.0
$\alpha$ -Carotene	Chromatographic separation of commercial carotene	7.6
Lycopene*	Tomato	31
Cryptoxanthin†	Yellow corn meal	trace
Xanthophylls‡	Spinach	trace

\* Isolated by the method of Strain (24).

† Isolated by the method of Fraps and Kemmerer (25).

‡ Extracted from the pigment-containing petroleum ether fraction with 90% methanol.

carbon carotenoids were most active; their oxygenated derivatives possessed only a trace of activity. All of the pigments were inactive for *L. casei*.

In Fig. 3 dose-response curves for the various types of substances which were active for *L. arabinosus* are given.

## DISCUSSION

The ability of representatives of the related types of compounds—fatty acids, keto acids, sterols, bile acids, sex hormones, saponins, heart poisons, resin acids, fat-soluble vitamins, terpenes and carotenoids—to replace acetate in varying degrees in its growth-stimulating capacity for the different species of lactic acid bacteria indicates that acetate may be utilized by these organisms for the synthesis of lipoidal materials. It is possible, of course, that the organisms transform each of the active compounds (with varying degrees of success) to some single substance essential for growth, which may or may not be lipoidal in nature. The non-saponifiable portion of *L. arabinosus* was more effective in replacing sodium acetate for this organism than any of the fat-soluble materials tested, a finding which is consistent with the proposed role for sodium acetate. In any case, the positive response of *L. arabinosus* to every type of compound listed may be interpreted

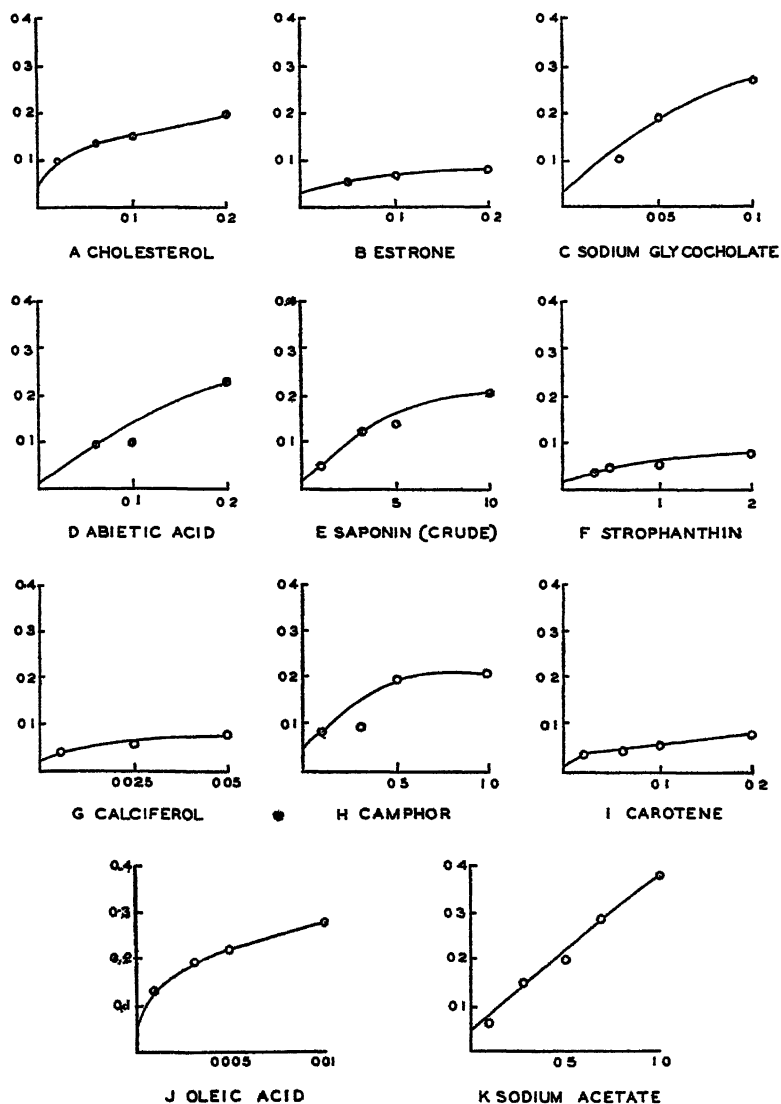


FIG. 3

Representative Dose-Response Curves for Various Types of Substances  
Which are Active for *L. arabinosus*

Weights of dry cells (mg./cc.) are plotted on the ordinates and corresponding weights of test substance (mg./10 cc. of culture medium) on the abscissae

as evidence that these structurally related compounds are also physiologically related. The inability of some of the other species of lactic acid bacteria to utilize many of these compounds may be due either to differences in the ability to assimilate them from the medium, arising, for example, from a difference in permeability of cell membranes, or to more fundamental differences in the metabolic utilization of the various compounds.

### SUMMARY

A marked effect of sodium acetate in stimulating early growth of lactic acid bacteria has been demonstrated. This effect is not given by other buffers and demonstrates functions for sodium acetate in bacteriological media other than that of buffer.

Various compounds belonging to the following classes were found capable of duplicating, in whole or in part, this growth-promoting action of acetate: fatty acids, keto acids, sterols, sex hormones, bile acids, saponins, heart poisons, resin acids, fat-soluble vitamins, terpenes and carotenoid pigments. The activity of these compounds suggests a probable role for acetate as a precursor for various lipids and may be interpreted as evidence for the physiological relationship of these structurally related compounds.

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# The Nutritional Role of Acetate for Lactic Acid Bacteria

## II. Fractionation of Extracts of Natural Materials

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### INTRODUCTION

In a previous communication (1) the response of lactic acid bacteria to substances reportedly involved in acetate metabolism of various organisms and tissues was given. Along with these substances, various cellular extracts were tested for their ability to duplicate the growth-stimulating effect of acetate for lactic acid bacteria. Many of these materials were more active for certain of the organisms than an equal weight of sodium acetate. Since their activity could not be due to acetate alone, the concentration of the active factor(s) in the most promising source materials was undertaken.

### EXPERIMENTAL

*Assay Procedure.* The medium and procedure were the same as that described in the previous paper (1). *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5 and *Lactobacillus acidophilus* were the organisms employed.

### RESULTS

*Substitution of Extracts of Various Natural Materials for Acetate.* Table I gives the response of the lactic acid bacteria to extracts of materials of natural origin.

Dehydrated grass juice, liver concentrate 1-20, liver fraction B, brewers' yeast extract, and Bacto yeast extract were more active for *L. casei* than an equal weight of sodium acetate. It might be assumed that these materials furnish this organism directly with some essential

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substance(s) for which acetate may serve as precursor. The growth stimulation produced by these materials is not due to the presence of any of the known water-soluble growth factors for microorganisms, since those nutrilites which were not already present in the medium were tested and found inactive in stimulating growth above the controls. Although some of the oils possessed greater than unit activity<sup>1</sup> for the organisms tested, they were not fractionated.

In addition to the substances listed above, various extracts of brewers' whole yeast were prepared, the activities of which were com-

TABLE I

*The Response of L. casei and L. arabinosus to Extracts of Natural Materials*

Substance tested	Activity (acetate equivalents/mg.) for	
	<i>L. casei</i>	<i>L. arabinosus</i>
Beef Extract	0.1	0.05
Cod Liver Oil	2.4*	1.2*
Cottonseed Oil	1.4*	0.43*
Curbay, B-G.	0.33	—
Dehydrated Grass Juice	10.0	0.08
Halibut Liver Oil	2.2*	1.8*
Liver Concentrate 1-20	1.7	0.1
Liver Fraction B	3.0	0.03
Peptone (Bacto)	1.0	0.05
Vacatone	0.33	0.21
Vitab	1.0	0.12
Wheat Germ Oil	1.5*	3.6*
Yeast Extract (Brewers')	10.0	0.08
Yeast Extract (Bacto)	10.7	0.13

\* Values expressed as acetate equivalents/0.001 cc.

pared with each other and with commercial yeast extracts. The results of this experiment are given in Table II. The difference in behavior of the two organisms is striking. *L. arabinosus* seems to respond primarily to fat-soluble substances while *L. casei* seems to respond primarily to one or more water-soluble substances. In this connection, it will be recalled (1) that whereas a great variety of fat-soluble substances were highly active in replacing sodium acetate for *L. arabinosus*, only a few of these showed similar activity for *L. casei*.

<sup>1</sup> One mg. of sodium acetate was assigned an activity of 1.0 (see preceding paper (1)).

*Concentration of the Active Water-Soluble Factor(s).* To follow the active water-soluble factor(s) during concentration *L. casei* was used as the test organism, as it was most sensitive to the active material in aqueous extracts. The active factor(s) was concentrated from each of the following source materials: dehydrated grass juice (Cerophyll Laboratories), brewers' yeast extract (Fleischmann's Type III), Bacto yeast extract (Difco), Curbay B-G. (U. S. Industrial Alcohol Co.), and Vitab (Vitab Corporation). The active fraction from each of these materials had many properties in common. It was, therefore, assumed that the same factor(s) was responsible for the activity in each case. Since the greatest concentration was obtained with brewers' yeast

TABLE II

*Comparison of Extracts of Yeast in Stimulating Growth of L. arabinosus and L. casei*

Description of Extract	Activity (acetate equivalents/mg.)	
	<i>L. arabinosus</i>	<i>L. casei</i>
1. Hot water extract of Whole Brewers' Yeast	0.14	2.4
2. Ether extract* of Whole Brewers' Yeast	7.0	3.0
3. Acetone extract* of Whole Brewers' Yeast	25.0	1.67
4. Hot water extract of residue from ether extraction	0.08	0.41
5. Brewers' Yeast Extract (Fleischmann's Type III)	0.06	10.0
6. Bacto Yeast Extract (Difco)	0.04	5-10

\* Extraction was carried out in a Soxhlet apparatus.

extract, only the experiments performed with this source material will be described.

Brewers' yeast extract was approximately 10 times as effective, weight for weight, as sodium acetate in stimulating growth of *L. casei*. The comparative response of the organism to the two substances is illustrated in Fig. 1. The most effective procedure developed for concentrating the active material from brewers' yeast extract is described below.

Dried brewers' yeast extract (6.6 g.) was placed in the extraction thimble of a Soxhlet extractor, then extracted with methanol for 6 hours. Ninety %<sup>2</sup> of the active substance but only 43% of the solids were removed by the methanol. An aliquot of the extract (182 cc.; 2.7 g. solids) was evaporated to a small volume, then diluted with water to 100 cc. Without adjusting the pH, 2.2 g. of activated charcoal (Darco

<sup>2</sup> All calculations were based on the activity of a freshly prepared solution of the original material. One mg. of this material was assigned an activity of 1.0.



(G-60) were added, and the mixture stirred for 15 minutes. The charcoal was filtered out, washed with water, and the active material eluted from it by refluxing for 10 minutes with 50 cc. of a 2.8% solution of ammonia in 50% ethanol. The elution procedure was repeated once. The combined eluates (134 cc.) contained 86% of the activity of the original yeast extract; the active material had been concentrated 18-fold at this stage (total weight: 295 mg.). The eluate was now evaporated on the steambath until only slightly alkaline, diluted to 50 cc. and the pH adjusted with sulfuric acid to 3.0. Two hundred thirty mg. of Darco G-60 were added, the mixture was stirred and the charcoal eluted in the same manner described above. The eluate

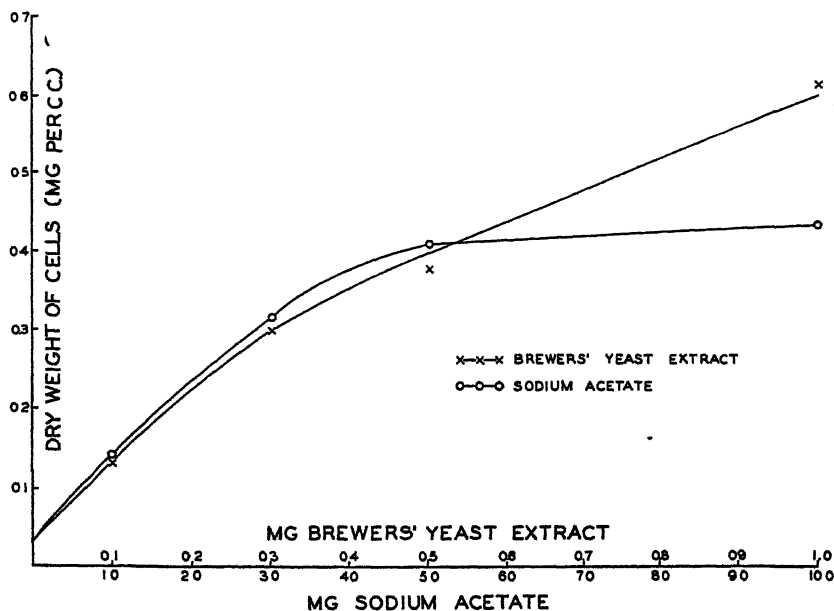


FIG. 1

Comparative Response of *L. casei* to Brewers' Yeast Extract and to Sodium Acetate

contained 49% of the activity of the original yeast extract and had been concentrated 44-fold (total weight: 67.2 mg.). A third adsorption and elution from charcoal did not effect further purification. Several separate concentrates were prepared at different times by the above procedure with only minor variations in the activity and amount of the end product obtained.

Although the active material in the yeast extract appeared stable to acids and alkalis at room temperature, it was partially destroyed by autoclaving aqueous solutions, either alone or with acid or alkali. Data from a typical experiment are given in Table III. Concentrates

of the active material were likewise stable to dilute acids at room temperature but were partially inactivated by standing for 24 hours in dilute alkaline solution (0.15 *N* sodium hydroxide). The activity of concentrates was not adversely affected by exposure to bright diffuse daylight for a period of 3 days.

The activity of concentrates from brewers' yeast extract was destroyed by the action of oxidizing agents such as dilute hydrogen peroxide or bromine water at room temperature. That the active substance is labile to oxidation is also indicated by the gradual loss of activity which occurred when solutions containing the active substance were stored for long periods of time. The addition of antioxidants such as ascorbic acid or cysteine hydrochloride (which were without activity in the growth test) partially prevented such loss. The lability

TABLE III

*Stability of the Active Material in Yeast Extract to Autoclaving\* in Neutral, Acid, and Basic Solutions*

Solution	Loss of activity per cent
Neutral	27
3 <i>N</i> Sodium hydroxide	65
6 <i>N</i> Hydrochloric acid	81

\* Each solution was autoclaved 30 minutes at 15 pounds steam pressure

of the active substance is also indicated by the partial losses of activity which occurred whenever solutions containing it were evaporated to smaller volumes. The reasons for this loss in activity were not elucidated.

When subjected to fractional electrical transport in the apparatus described by Williams (2), the active factor(s) migrated to the anode, indicating that it is acidic. Only 30% of the original activity was recovered after such treatment, suggesting oxidative destruction at the anode.

The addition of 1 mg. of ascorbic acid/10 cc. of the ethanolic ammonia used for elution slightly improved the yield of active material and also permitted the eluate to be kept for a longer period of time without diminution in activity. Whenever ascorbic acid was added to the eluant, it was also included in the basal medium to the extent of 1 mg. per tube.

Pretreatment of charcoal with aniline, or with reducing agents, or

with potassium cyanide (3) failed to increase the activity of the eluates obtained. Superfiltrol and permutit were less satisfactory adsorbents than charcoal. The active substance was adsorbed on the Amberlite resins but could not be eluted from them by the procedures tried.

The active substance was partially or completely precipitated from solution by addition of soluble salts of silver, lead or mercury, and also by addition of phosphotungstic acid. Although the active material could sometimes be recovered from such precipitates, the procedures led to no purification of the active material and were therefore abandoned.

### SUMMARY

The occurrence in materials of natural origin of a water-soluble substance (or substances) which replaces acetate in its growth-promoting function for some lactic acid bacteria, and which is much more active than acetate on an equal weight basis, was demonstrated.

A procedure for concentrating the active factor(s) from the most promising source materials is described. A typical concentrate from brewers' yeast extract was 44 times as active as the starting material; 1 mg. of this material was equivalent in growth-promoting action to 440 mg. of sodium acetate.

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# The Role of Corn Steep Liquor in the Production of Penicillin \*

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## INTRODUCTION

Corn steep liquor is the water extract obtained in the industrial manufacture of starch, gluten and other corn products. It is usually concentrated to about 50% solids and is used chiefly in the manufacture of commercial feeding stuffs. It has recently attracted much attention from another point of view, *viz.*, penicillin production by molds. In a recent review Coghill (1) has credited A. J. Moyer with discovering the usefulness of corn steep liquor for penicillin production. The addition of corn steep liquor to the medium increased the penicillin yield at least tenfold above the previously reported yields. Although some synthetic media have been developed which give fairly good yields of penicillin, the simplest and most practical way of obtaining optimum yields is by the addition of corn steep liquor to the medium. Since the corn steep liquor stimulates penicillin production even after proper adjustment of the medium constituents, pH and aeration, the most reasonable explanation of its effectiveness is that it contains some precursor or key substance which is used by the mold in producing penicillin.

Other materials may be used in place of corn steep liquor in the production of penicillin. Halpern *et al.* (2) have reported stimulation of growth and production of penicillin by *P. notatum* 832 by the butyl alcohol-soluble fraction of wheat extracts and protein hydrolyzates. Proline together with glutamic acid gave 35 Oxford units of penicillin per ml. of medium. These yields are far below the highest yields obtained

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with corn steep liquor. Cook, Tulloch, Brown and Brodie (3, 4) have reported good yields of penicillin using aqueous extracts of peas instead of corn steep liquor. The hot aqueous extract gave as high as 225 units of penicillin per ml., and the 80% alcohol-insoluble portion of this extract gave 200 units per ml. with surface cultures. According to White, Krampitz and Werkman (5), 205 units of penicillin may be obtained with a combination of arginine, histidine, glutamic acid and succinic acid in place of corn steep liquor in surface cultures. In industrial practice, as far as the authors are aware, no substitute has proved as efficient and economical as corn steep liquor.

### EXPERIMENTAL PROCEDURE

*Cultures.* Two species and several strains of penicillia were used, *P. notatum* 832 and *P. chrysogenum* NRRL 1951-B25, 35217, 45417, and X-1612. Cultures 832 and 1951-B25 were obtained from Dr. R. D. Coghill of the Northern Regional Research Laboratory. Cultures 35217 and 45417 were obtained from Dr. G. W. Beadle of Stanford University, and were derived by the Stanford workers from 1951-B25 after irradiation of the parent culture with ultraviolet light. Culture X-1612 was derived from culture 1951-B25 by irradiation of the parent culture with x-rays. The irradiation and isolation work was done by Dr. M. Demerec, Carnegie Institution, Cold Spring Harbor. The organism was sent to us by Dr. C. M. Christensen of the University of Minnesota for the determination of its penicillin-producing ability in submerged fermentations in tanks.

*Medium and Fermentation.* The majority of runs reported in this study were performed with shaken flask cultures. Medium I, which was originally developed by the Northern Regional Research Laboratory, consisted of 2% lactose, corn steep liquor equivalent to from 1 to 4% solids (see Tables), 0.05%  $\text{KH}_2\text{PO}_4$ , 0.3%  $\text{NaNO}_3$ , 0.004%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.025%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . One hundred ml. of medium were placed in 500 ml. Erlenmeyer flasks, sterilized, inoculated and aerated at 23°C. in reciprocating shakers operating at 80 to 90 10-cm. strokes per minute. The medium was inoculated with a two day-old vegetative cell inoculum which was prepared in the following manner: A bottle plate containing a spore-producing medium (II) devised by the Northern Regional Research Laboratories (peptone, 5 g.; Brer Rabbit molasses, 7.5 g.; residue from alcohol fermentation of molasses (Curbay BG), 2.5 g.; glycerol, 7.5 g.;  $\text{NaCl}$ , 4 g.;  $\text{KH}_2\text{PO}_4$ , 0.06 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05 g.; agar, 15 g.; distilled water to 1 liter) was inoculated with a soil stock of the culture and incubated at 23°C. for two days. The spores were then suspended in 50 ml. of water and 2 ml. were added to a 500 ml. Erlenmeyer flask containing 100 ml. of a 6% dextrin (No. 151 Corn Products Refining Company), 2% corn steep solids, and salts (Medium III). This medium was incubated at 23°C. for two days with shaking. Two ml. of this medium were then used to inoculate each of the flasks used in the runs.

*Penicillin.* The amount of penicillin in the fermented medium was determined by an assay procedure based on the method of Schmidt and Moyer (6) with *Staphylococcus aureus* as the test organism. A penicillin preparation obtained from the Abbott Laboratories was used as a working standard. This was checked against a standard obtained from the Food and Drug Administration, Washington. The penicillin yields are expressed in Oxford units.

*Corn Steep Liquor.* In the preliminary work more than 40 samples of steep liquor from six companies were tested. It soon became evident that not all of these could be tested from various angles; hence, the later work was confined to a much smaller number of samples from fewer companies. Analytical data from the producers showed that the corn steep liquor samples consisted of 40–60% solids and contained, on the dry basis, 12 to 27% lactic acid, 7.4 to 7.8% total nitrogen, 2.6 to 3.3% amino nitrogen, 1.5 to 14% reducing sugars calculated as glucose, and 18 to 20% ash.

### REPRODUCIBILITY OF FERMENTATIONS

A great deal of difficulty has been experienced in reproducing the yield of penicillin in successive experiments. In the usual fermentation,

TABLE I

*Reproducibility of Penicillin Production by P. chrysogenum X-1612*  
Medium I with 2% corn steep

Run No.	Penicillin Units per ml. after						pH after .					
	4 days	5 days	6 days	7 days	8 days	9 days	4 days	5 days	6 days	7 days	8 days	9 days
1	60	83	53*	114		76	7.2	7.1	7.6	7.6	8.0	8.2
2		96	97	69	103*	64	6.8	7.5	8.0	8.2	8.4	8.5
3	87	112*	83	78	95	75	7.1	7.3	7.6	7.9	8.1	7.9
4	60	109	91	94	86	80	7.2	7.4	7.7	8.0	8.2	8.4
5	80	96	79	54	56	63	7.0	7.3	7.9	8.0	8.4	8.4
6	42	52	52	60	68	90	6.7	7.1	7.6	7.6	7.7	8.1

\* These values are out of line with the other penicillin figures in the series and also with the pH data. They should not be given the same significance as the other values.

the penicillin content of the medium rises to a maximum and then falls off rapidly. In successive experiments, cultures do not develop at exactly the same rate, hence the day of maximum yield may vary from fermentation to fermentation. The best single index of the course of the fermentation other than the content of penicillin is the pH of the medium. With culture X-1612 about 110 units per ml. have been obtained in six successive runs although the day of the maximum yield and pH on this day show some variations (see Table I). The maximum concentration of penicillin in the medium was found to occur any time between the fifth day and the eighth day of the fermentation. The pH

during the time of maximum yield was usually between 7.0 and 8.0. It is very important that the pH of the culture be maintained at its proper value since little penicillin is produced if the pH rises too rapidly to 8.0 or above. A more detailed discussion of the relationship between pH and penicillin production is presented in the paper by Koffler, Emerson, Perlman and Burris (7).

### OPTIMUM LEVEL AND VARIABILITY OF CORN STEEP LIQUOR

Table II gives typical data obtained in testing various levels of corn steep liquor in shaken flasks. In most cases the optimum level of corn steep liquor was found to be that equivalent to 2% solids in the

TABLE II  
*Penicillin Production at Various Levels of Corn Steep Liquor*  
Medium II

Experiment No	Corn steep liquor used		Culture 832			Culture X-1612		
	Number (and producer)	Per cent Dry solids	Day of maximum yield	Penicillin u/ml.	pH at maximum	Day of maximum yield	Penicillin u/ml	pH at maximum
1	38 (A)	1	9	38	7.8	9	103	8.3
2	38 (A)	2	9	65	8.0	9	148	8.3
3	38 (A)	4	6	62	8.1	9	117	8.3
4	40 (A)	1	7	45	7.7	9	94	8.5
5	40 (A)	2	7	72	7.5	9	142	8.4
6	40 (A)	4	7	49	8.0	9	144	8.2
7	39 (B)	1	8	48	8.2	9	89	8.4
8	39 (B)	2	7	58	7.7	9	129	8.4
9	39 (B)	4	6	31	8.1	9	109	8.3
10	64 (B)	1	7	97	7.9	6	113	7.1
11	64 (B)	2	7	164	7.7	11	174	8.5
12	64 (B)	4	6	84	7.7	11	206	8.2
13	49 (C)	1	8	42	8.4	9	133	8.4
14	49 (C)	2	7	48	8.4	9	101	8.4
15	49 (C)	4	7	34	8.2	9	93	8.2
16	65 (C)	1	8	107	7.6	7	166	7.2
17	65 (C)	2	7	100	7.7	7	160	7.5
18	65 (C)	4	7	69	7.6	12	154	7.9
				Av. 67			Av. 132	

medium. The superiority of the 2% level was much more marked with culture 832 than with X-1612. With the former the 2% level gave about 60% more penicillin than either the 1 or 4% levels. With the latter there was no difference between the 2 and 4% levels but both were nearly 50% better than the 1% level. Fermentations with 20 other lots of corn steep with culture 832 showed that the 2% level was as good as or better than the 4% level and both were much better than the 1% level. As will be shown later, the optimum concentration in stirred and aerated tanks is not the same as that in shaken flasks. Different lots of corn steep showed great variation in stimulating the production of penicillin. Lot 64 gave three times the yield of Lot 49 with culture 832 and about one-third more with X-1612. The variation was not related to the source of the steep as there was about an equal difference between Lots 39 and 64, which were made by the same manufacturer as between Lots 64 and 49 which came from different producers. Corn steep liquor is certainly not a standard product as far as production of penicillin is concerned. Even if it were standardized for one method of fermentation, *e.g.*, shaken flasks, it by no means follows that uniform results would be obtained in tank fermentations. Of course, it must be recognized that factors other than corn steep liquor operate to affect the yield of penicillin. Some of these are inherent variations in the mold itself; others are due to external conditions such as pH and aeration. Many more data must be accumulated before the exact role of corn steep liquor in penicillin production can be fairly assessed.

The superiority of culture X-1612 over 832 is shown in all of the experiments. Not only was it less affected by changes in the lots of corn steep but on the average it produced twice as much penicillin as 832. However, it took about 2 days longer to reach this higher level.

#### EFFECT OF ADDING CORN STEEP LIQUOR TO A MEDIUM PROVIDING GOOD GROWTH WITHOUT IT

That it is not enough to maintain proper pH and mycelial growth to obtain maximum penicillin is illustrated by the data presented in Table III. A chemically defined medium (IV) developed by other members of this laboratory to produce as good mycelial growth as was obtained on Medium I, and also to maintain pH conditions similar to those obtained on Medium I was used as a basal medium. It contained



2% lactose, 2% dextrin, 0.82% ammonium lactate, and the same concentration of salts as in Medium I. Since lactic acid is fermented readily by the mold, the ammonia liberated tends to counteract the acid produced by the fermentation of the lactose and dextrin. When the basal medium was used alone, no penicillin was produced although

TABLE III

*Stimulation of Penicillin Production by Corn Steep Liquor in Media Giving Good Mycelial Growth*

Experiment No	Medium	Penicillin		Organism	pH on day of maximum yield	Weight of mycelium after 9 days, mg/ml of medium
		Day of maximum yield	u/ml			
1	I, 2% C.S.L.	8	61	832	7.6	10.1
2	IV	—	0	832	—	12.6
3	IV+0.1% C.S.L. No. 64	9	20	832	8.0	14.1
4	IV+1.0% C.S.L. No. 64	7	40	832	8.1	17.0
5	IV+2.0% C.S.L. No. 64	7	56	832	8.2	17.9
6	IV	—	0	X-1612	—	13.1
7	IV+0.1% C.S.L. No. 64	7	48	X-1612	7.9	11.5
8	IV+1.0% C.S.L. No. 64	9	72	X-1612	8.3	13.2
9	IV+2.0% C.S.L. No. 64	9	95	X-1612	8.1	15.3
10	I+2% C.S.L. No. 64	9	127	X-1612	8.2	10.6

the mycelial growth was even better than that produced with the regular medium in which the greatest amount of penicillin was obtained. Addition of corn steep liquor to this medium considerably increased the penicillin-producing capacity.

#### PENICILLIN-PRODUCING CAPACITY OF STEEPWATER FRACTIONS

A number of steep liquors were prepared by the Corn Products Refining Company by extracting the same batch of corn with successive volumes of SO<sub>2</sub>-treated water to determine at what period the active material in the corn was extracted. The extracts were concentrated in glass equipment and sent to our laboratory to be tested for penicillin-producing capacity. The results are presented in Table IV. Most of the active material appears to be extracted within the first

TABLE IV  
*Penicillin-Producing Capacity of Steepwater Fractions*  
 Medium I

Experiment No.	Corn steep fractions		Penicillin		pH on day of maximum yield
	Hours of steeping	Per cent solids used	Day of maximum yield	u./ml.	
1	0-24	2	7	56	7.7
2	24-48	2	7	25	7.7
3	48-72	2	8-9	24	7.8
4	72-96	2	8-9	18	7.8
5	96-120	2	9	18	7.9
6	120-168	2	6	22	7.5
7	168-192	2	9	26	7.6
Control		2	7	44	8.0

24 hours, as the yield of penicillin with this fraction was more than twice as great as that produced with any of the other fractions.

#### LABORATORY-PREPARED CORN STEEP LIQUORS

Several types of steep liquors were prepared in the laboratory by the following method: 250 g. of corn meal were suspended in 500 ml. of distilled water and extracted at 65°C. for 24 hours with occasional shaking. Several varieties of steeps were prepared in this manner. Corn meal was extracted at pH values of 3.0, 6.0 and 9.0. In some cases 0.24%  $\text{Na}_2\text{SO}_3$  was added to the suspension and in other cases the corn was sprouted for four days previous to grinding and steeping. After the steeping process, the corn meal was filtered off and the liquid portion was used in the penicillin medium. The weight of dry solids in the corn steep liquor made from unsprouted corn was about 3% while that made from sprouted corn contained about 10%.

The steep liquors were used at a level of 2% solids in all the fermentations. The results of these fermentations are summarized in Table V. The extracts from unsprouted corn meal at pH values of 3.0 and 9.0 were very poor, yielding only 16 or 17 units of penicillin per ml. The steep liquors extracted at pH 6.0 occasionally were as good as commercial corn steep liquor (yielding 48 units of penicillin per ml.). The best results were obtained with the steep liquors prepared from sprouted corn. Addition of 0.24%  $\text{Na}_2\text{SO}_3$  to the steeping water seemed to improve the effectiveness of the product in most cases. Penicillin yields which were consistently as good as those obtained with the commercial steep liquors were obtained with these steeps.

TABLE V  
*Potency of Laboratory Prepared Corn Steep Liquor*  
 Medium I

Experiment No.	Variable constituents of the medium		Penicillin		pH on day of maximum yield
	Kind	Per cent Solids used	Day of maximum yield	u./ml.	
1	Corn steep liquor No. 2	2	7	48	8.0
2	Corn meal extract, pH 3, 30°C.	2	5	17	8.2
3	Corn meal extract, pH 6, 30°C.	2	6	9	8.0
4	Corn meal extract, pH 9, 30°C.	2	5	16	8.2
5	Corn meal extract, pH 6, 30°C., in presence of 0.24% $\text{Na}_2\text{SO}_3$	2	7	35	8.2
6	Corn meal extract, pH 6, 65°C., in presence of 0.24% $\text{Na}_2\text{SO}_3$	2	5	18	8.0
7	Corn meal extract, pH 3, 65°C.	2	7	14	8.5
8	Corn meal extract, pH 6, 65°C.	2	10	38	> 8.1
9	Corn meal extract, pH 9, 65°C.	2		0	
10	Corn meal extract, pH 6, 65°C., in presence of 0.24% $\text{Na}_2\text{SO}_3$	2	6	21	8.0
11	Germinated corn extract, pH 6, 65°C.	2	8	24	8.2
12	Germinated corn extract, pH 6, 65°C., in presence of 0.24% $\text{Na}_2\text{SO}_3$	2	9	53	8.4

### FERMENTED STEEPS

More or less fermentation takes place in processing corn steep liquor. This fermentation is largely due to lactic acid-producing bacteria and mycoderms but yeasts may also play a part as they are usually found in the steep liquor. The effect of this microbial action on the penicillin-producing capacity of corn steep liquor is not definitely known. A yeast type of fermentation is regarded by some producers as undesirable. A series of steep liquors which had been incubated at temperatures ranging from 38°C. to 54°C. was sent to us by the Corn Products Refining Company. The lactic acid content of these steep liquors ranged from 23.5–28% on the dry basis. No appreciable difference was noticed between the steep liquors when they were tested for their penicillin-producing capacity nor did there seem to be any correlation

between the amount of lactic acid contained in the steep liquor and the penicillin produced.

Some samples of steep liquor were deliberately inoculated with *Saccharomyces cerevisiae* and incubated with aeration at 30°C. for 24 hours, sometimes with 1% added glucose. This procedure produced a heavy growth of yeast cells in the steep liquor and improved the penicillin yield as much as 20% in some instances. Yeast grown in steep liquor without aeration had little or no effect. The addition of 1% yeast

TABLE VI  
*Effect of Supplements of Natural Materials on Penicillin Production*  
Medium I

Experiment No.	Variable constituents of the medium		Penicillin		pH on day of maximum penicillin yield
	Kind	Per cent Dry solids	Day of maximum yield	u./ml.	
1	Corn steep liquor No. 38	2	5	51	7.7
2	Corn steep liquor No. 38	3	7	58	8.3
3	Corn steep liquor No. 38	2	6	61	8.3
	Unhydrolyzed casein	1			
4	Corn steep liquor No. 38	2	6	58	8.3
	Solubilized liver	1			
5	Corn steep liquor No. 38	2	6	54	8.2
	Bacto peptone	1			
6	Corn steep liquor No. 38	2	5	59	7.8
	Difco yeast extract	1			
7	Corn steep liquor No. 38	2	6	63	8.2
	Grass juice powder	1			
8	Corn steep liquor No. 38	2	6	70	8.1
	Potato tuber tissue	1			
9	Corn steep liquor No. 38	2	7	62	8.1
	Milk powder	1			
10	Corn steep liquor No. 38	2	4	66	7.7
	Fish meal	1			
11	Corn steep liquor No. 38	2	6	79	8.5
	Meat scraps meal	1			
12	Corn steep liquor No. 38	2	5	69	8.9
	Soy bean meal	1			
13	Corn steep liquor No. 38	2	6	63	7.3
	Potato extract	1			
14	Corn steep liquor No. 38	2	7	63	8.2
	Asparagus butt juice	1			

cells (dry basis) without fermentation also seemed to improve the potency of the steep by about 10%. (Data are not recorded in tables.)

### CORN STEEP LIQUOR SUBSTITUTES AND SUPPLEMENTS

No satisfactory substitute for corn steep liquor has been found. The following substances have been tested and found to be of little value as substitutes for corn steep liquor: Cuban high test molasses, steffanized molasses A and B, Bacto peptone, solubilized liver, asparagus-butt juice, Difco yeast extract, grass juice powder, rice steep,

TABLE VII

*Penicillin Production in Aerated and Stirred Media (9 l. bottles)*

Experiment No *	Corn steep liquor		Per cent Lactose	Penicillin		pH on day of maximum	Number of organisms
	Number	Per cent Dry solids		Day of maximum yield	u/ml		
1	54	2	2	6	194	7.3	35217
2	54	4	3	6	148	7.0	35217
3	54	2	2	6	169	7.5	35347
4	54	4	3	6	207	7.6	35347
5	54	2	2	6	159	7.9	45417
6	54	4	3	6	209	7.6	45417
7	2	2	2	6	68	7.6	832
8	51	2	2	7	90	7.7	832
9	51	4	3	6	145	7.4	832
10	63	4	3	5	223	6.5	X-1612

\* Medium I with corn steep and lactose as noted.

cottonseed meal extract, ground liver and liver infusion. Meat scraps meal was somewhat better than most of these but not as good as corn steep.

The yield of penicillin could be increased, however, by the addition of 1% of several natural materials to the lactose-corn steep-salts medium. The data presented in Table VI show that unhydrolyzed casein, Bacto peptone, Difco yeast extract, grass juice powder, potato tuber tissue, milk powder, fish meal, meat scraps meal, soy bean meal, potato extract and asparagus-butt juice increased the yield of penicillin 5 to 50% above that produced on the regular medium. Defatted

hydrolyzed corn germ, hydrolyzed whole wheat germ, hydrolyzed zein, and hydrolyzed casein, and defatted hydrolyzed sunflower meal decreased the yield 2-35% when added as a supplement to the medium.

#### AERATED AND STIRRED FERMENTATIONS IN BOTTLES AND TANKS

In Tables VII and VIII representative data are recorded for runs made with larger volumes of media. One type of apparatus used for fermenting larger volumes of media was a glass bottle having a capacity of 9 liters. Four liters of medium were added and constantly stirred and aerated during the process of fermentation. An average

TABLE VIII

#### *Penicillin Production in Tank Fermentations*

Experiment No *	Organism	Per cent Corn steep solids dry solids	Per cent Lactose	Penicillin		pH on day of maximum yield
				Day of maximum yield	u/ml	
1	X-1612	2	2	2	240	8.0
2	X-1612	4	3	3	480	7.3
3	X-1612	6	3	3	636	7.6
4	832	2	2	3	62	7.5
5	832	2	1	3	37	8.0
6	1951-B25	4	3	4	126	7.4
7	832	4	3	3	44	8.2

\* Medium I with corn steep and lactose as noted.

rate of 16 liters of air per minute was used. Still larger quantities of medium were handled in 80-gallon tanks in which 220 liters of medium were fermented with aeration as high as 200 liters per minute. Details on construction and operation of these fermentations under various conditions will be presented elsewhere.

Contrary to the data obtained with shaken flasks, the optimum level of corn steep liquor in large volume fermentations was 4% on the dry basis. The maximum yield of penicillin was obtained on the sixth day with bottles and on the third day with tanks as compared with the seventh to ninth day with shaken flasks. These differences are probably due to the effect of increased aeration in the bottles and tanks. The variation between runs in large volume fermentations was less than

that of shaken flasks. This may have been due to the better control of agitation in the bottles and tanks.

#### ACKNOWLEDGMENT

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#### SUMMARY

Corn steep liquor in the medium seems necessary for optimum yields of penicillin. No satisfactory substitute for corn steep liquor was found among more than 20 natural materials tested. However, some of these, particularly meat scraps meal and potatoes, supplemented the action of corn steep liquor in increasing the yield of penicillin.

Samples of corn steep liquor from different manufacturers varied greatly in their penicillin-producing capacity. However, the variations were as great in lots from the same manufacturer as in lots from different manufacturers. Fermentation of the corn steep liquor with yeast seemed to increase rather than decrease the penicillin-producing potency of the steep liquor.

The optimum level of corn steep solids in shaken flasks was found to be 2% when either *P. notatum* 832 or *P. chrysogenum* X-1612 were used. Data obtained with larger volumes of medium under aeration and agitation indicated that 4% corn steep solids was optimal.

The most potent steep prepared in the laboratory was obtained from sprouted corn, extracted at 65°C., pH 6, in the presence of 0.24% Na<sub>2</sub>SO<sub>3</sub>. The most active part of the steep liquor was extracted from the corn during the first 24 hours.

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# Relationship Between the Dose of Factor N and the Alcohol Intake of Rats Under Self-Selection Conditions

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## INTRODUCTION

In previous papers we have pointed out that rats receiving alcohol under conditions of self-selection, drink more alcohol when a thermolabile factor present in yeast is missing from their diet. This substance we have called "Factor N"\* (1). When rats receive yeast, liver, meat or wheat germ together with the deficient diet, the increased intake of alcohol is markedly reduced (2). We have also observed that thiamin, riboflavin, pyridoxine, calcium pantothenate, niacin, inositol, choline or liver extract containing biotin, when given in adequate doses, either alone or as mixtures, do not reduce the alcohol intake of deficient rats (3, 4). We have not tried folic acid, but we have observed that factor N is not adsorbed by Norit A. Hence, we have claimed that factor N is a new member of the B-complex.

Obviously we must establish a working unit for factor N. For various reasons we have defined this working unit as: "That quantity of factor N producing a reduction in the average alcohol intake per 100 g. body weight of animal during the five days following the first dose, equal to 50% of the extra intake during the five preceding days, if given daily, *per os*, for three consecutive days to each rat of an

\* When this paper was in press we knew of a paper by R. M. Calder (*J. Path. Bact.* 54, 355 1942) in which he pointed out the existence of a thermolabile factor in the vitamin B complex, which he also called "factor N," whose presence in the diet favors the toxic effect of chloroform on the liver of rats under certain experimental conditions. There is no reason to consider Calder's "factor N" and our "factor N" the same substance, although both are thermolabile factors of the vitamin B complex other than thiamine.

experimental group whose alcohol intake has attained a regular level higher than 0.5 cc./100 g./day because of the restricted diet."

To compute the unit value when the assay shows effects higher or lower than that of one unit, we have used an empirical formula, deduced experimentally, until a more extensive study allows obtainance of a better expression (5).

We are now able to obtain a sufficiently stable liver extract by the following procedure:

*Extract BH S.01.* Fifty kg. of raw mashed beef liver are mixed with 100 l. of water containing 330 ml. of sulphuric acid at pH 2. The mixture is heated to 65°C. and sufficient  $\text{Ca}(\text{OH})_2$  immediately added to bring the pH to 5.0. The solids are separated by filtration and the liquid phase concentrated *in vacuo* and desiccated by spraying. Yield, 640 g.

If we assume the initial activity of liver used in this preparation to be equal to the average of the results of our previous testing of 10 different samples of raw beef liver (unpublished data), *i.e.*,  $0.27 \pm 0.07$  units per g., the total activity would have been  $13,500 \pm 3500$  units; the actual yield was  $640 \times 2.6$  (see below) = 1664 units, *i.e.*, 12%. As a point of comparison, tests on 12 samples of dried yeast (*Torula utilis*) has shown an activity of  $1.29 \pm 0.23$  U. per g.

The present paper deals with the results obtained with varying doses of this liver extract on different groups of rats (27 determinations).

## PROCEDURE

Experimental animals are white rats bred in our laboratory, weighing 80 to 100 g. Each group of 3 to 6 rats was placed in a screen-bottom metal cage to prevent access to excreta. Rats have access to distilled water and to 10 and 20% by volume alcohol solutions placed in inverted graduated cylinders. The alcohol intake and animals' weight were measured daily.

The deficient diet is as follows:

Commercial casein <sup>a</sup>	20 parts
Sucrose	60
Vegetable oil enriched in vit. A and D <sup>b</sup>	15
Dry treated brewer's yeast <sup>c</sup>	10
Salt mixture (Osborne and Mendel)	5

<sup>a</sup> Commercial casein is free from factor N (6).

<sup>b</sup> Vegetable oil containing 60 units of vitamin A and 10 units of vitamin D per cc. added in the form of Haliverol, Parke Davis.

<sup>c</sup> Dry brewer's yeast autoclaved at 125°C., for 90 minutes at pH 9.

Alcohol intake, under these self-selection conditions, rises regularly during the first 15 to 30 days and is then stabilized at 0.5 to 1.0 cc. of ethyl alcohol per 100 g. body weight per day. When stabilization is reached, the group is ready for addition, *per os*, of the substance to be tested. Since the effect of three day dosage with factor N only lasts for a few days, the group is ready for another test run after alcohol intake has been stabilized again at high level. Control animals show an alcohol intake ranging from 0.05 to 0.20 cc. of ethyl alcohol per 100 g. body weight per day.

In the 27 experiments we have used 19 different groups of animals.

# CALCULATIONS

It is first necessary to establish the effect of the added material on the substance used to compute the unit value. Fig. 1 shows the result

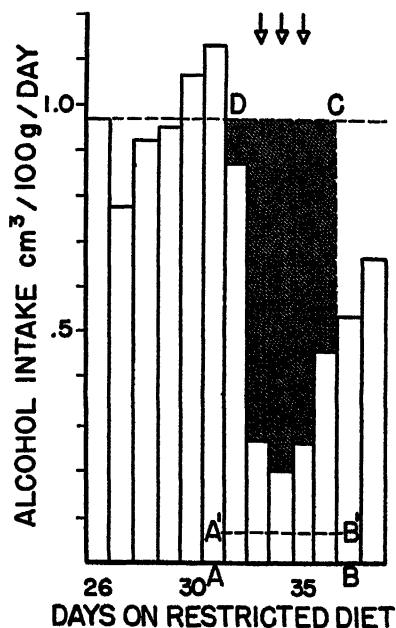


Fig. 1. Effect of Administration of Liver Extract on the Alcohol Intake of Deprived Rats. Arrows: 0.5g liver extract BH.S.01 *per os* to each rat.

of one assay. The shaded area represents the decrease in alcohol intake due to addition of the amount of factor N contained in 0.5 g. of liver extract.

The maximum theoretical decrease would be measured by the area ABCD. The actual effect is then also a part of the theoretical action,

and can be expressed by the relation between the shaded area and the area ABCD. But, as the maximum effect is never reached (*i.e.*, the alcohol intake never reaches 0), it seems better to use the lower level as a basis of comparison. The study of more than 300 assays of various substances shows that an alcohol intake of less than 0.05 cc./100 g./day is exceptional with rats on the unrestricted diet. Then area A'B'CD represents the maximum observed effect. On this basis, we express the activity of a given dose of a substance by the quotient between the observed decrement in alcohol intake and the maximum possible effect. Then, if we call  $a$ , the total alcohol intake during the five preceding days, and  $b$ , the total alcohol intake during the five days following the first dose, both expressed in cc./100 g./day, the activity will be

$$A = \frac{a - b}{a - (0.05 \times 5)}$$

Using this criterion we can study the relationship between the activity shown by a substance and its dose.

### RESULTS

Table 1 shows the result of the measurement of the activity of varying doses of our liver extract BH. S.01. Though the figures show appreciable fluctuations the relation between activity and dose is clearly linear. Statistical analysis, excluding the supramaximal value of the 1 g. dose, gives for the trend,  $y = 1.3x$ , and for the correlation coefficient,  $r = 0.934$ .

In this particular liver extract, the activity as computed from the above figures, is 1 unit in 0.38 g., *i.e.*, 2.6 units per g.

### DISCUSSION

The linear trend allows us to compute the units in a given substance by a simple formula. From the definition of the unit we have, for a dose equal to 1 unit, effect  $A = 0.5$ . Hence, the number of units contained in a given dose will be

$$2A = \frac{2(a - b)}{a - 0.25}$$

It is important to notice that, due to the fluctuations of the results,

TABLE I

Dose	Group number	Number of rats	Sex	Age at start of deprived diet	Days on deprived diet at start of test	Weight of Group		Alcohol Intake		$\frac{a-b}{a-0.25}$
						at start of test	at end of test	during 5 preceding days <i>a</i>	during 5 days following first dose <i>b</i>	
				<i>days</i>	<i>days</i>	<i>g</i>	<i>g</i>	<i>cc/100 g</i>	<i>cc/100 g.</i>	
0.08	13/25	4	♂	47	102	435	450	4.07	3.87	0.05
0.08	14/17	3	♂	63	70	328	340	3.34	2.82	0.17
0.08	19/25	5	♂	57	27	510	510	4.37	3.00	0.33
0.08	20/24	5	♂	55	38	625	600	3.07	2.95	0.04
									mean	0.15
0.20	15/4	5	♂	53	91	490	514	2.73	2.99	0.10
0.20	14/23	5	♀	53	91	456	498	3.46	1.14	0.72
0.20	15/4	4	♂	53	126	428	472	3.64	2.00	0.48
0.20	19/32	5	♂	55	56	530	560	4.01	2.32	0.45
0.20	19/25	5	♂	55	64	442	441	3.62	3.60	0.00
0.20	14/1	3	♀	60	177	437	462	3.71	2.39	0.38
0.20	20/5	5	♀	50	60	623	619	2.81	2.03	0.31
0.20	20/23	5	♂	49	55	717	682	2.84	1.58	0.49
									mean	0.34
0.33	14/24	3	♀	117	59	332	334	3.70	2.26	0.42
0.40	14/29	5	♀	65	61	532	605	3.61	1.66	0.58
0.40	15/4	5	♂	53	100	530	573	3.18	2.46	0.25
0.40	18/20	3	♂	51	73	383	433	4.59	1.03	0.87
0.40	20/17	6	♀	50	40	562	577	3.01	1.00	0.73
									mean	0.61
0.50	18/20	3	♂	51	38	398	451	4.84	2.08	0.60
0.50	20/33	6	♂	48	32	688	767	3.75	2.48	0.36
0.50	20/21	5	♀	38	50	495	570	2.56	0.72	0.89
0.50	20/33	6	♂	48	42	727	815	3.313	2.24	0.31
0.50	20/23	5	♀	54	64	525	593	5.93	1.14	0.58
0.50	19/25	5	♂	56	83	397	437	3.43	0.29	0.99
									mean	0.62
0.70	16/21	3	♂	42	49	295	338	2.70	1.04	0.68
0.70	18/31	5	♀	53	70	410	512	3.97	0.17	1.02
0.70	20/5	5	♀	50	40	607	670	2.47	1.12	0.62
0.70	19/34	5	♂	166	59	603	713	2.33	0.46	0.90
									mean	0.81
1.00	18/31	5	♀	53	39	466	561	4.68	0.46	0.95

it is advisable to use not less than three groups of rats and try doses the content of which is of the order of 1 to 2 units.

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# Enzymatic Nature of Cell-Free Extracts from Bacteria

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## INTRODUCTION

Wiggert *et al.* (1940) outlined a comparatively simple method for obtaining cell-free extracts from bacteria by grinding the cells with powdered glass in a mortar and pestle. Later, the method was modified and put on a semi-mechanical basis by Kalnitsky *et al.* (1945). During the last few years, the modified method has been successfully applied in this laboratory to a number of *genera* of bacteria and yeast. This report comprises a description of the extracts obtained, especially with regard to the nature and variety of substrates attacked by the preparations. In addition, extracts from two species have been studied in sufficient detail to permit the observation that the properties of the extracts are greatly affected by the growth conditions of the cells from which the extracts are obtained.

Although necessarily exploratory in nature, the data point the way to more detailed studies; and the preparations should prove especially valuable in investigations of mechanism where permeability difficulties are to be avoided.

## METHODS

The following medium has been satisfactory for the growth in large quantity of a wide variety of bacterial species: 1% glucose, 0.4% yeast extract, 0.8%  $K_2HPO_4$  and 10% tap water. The last two items were sterilized in a separate flask. Occasionally, it may be necessary to partially neutralize the  $K_2HPO_4$  before growth is initiated. Unless otherwise specified, the bacteria used in these experiments were grown in the above medium in ten-liter quantities for 20–40 hours at 30°C. The cells were harvested with the Sharples supercentrifuge and treated with ground glass according to the method described in detail by Kalnitsky *et al.* (1945). The extracts were clarified, following the grinding process, by centrifugation on a Beams air-driven ultracentrifuge (Beams, 1930) until reasonably clear preparations were obtained, although the time required to reach this state varied markedly with the species.



Experiments were conducted in the usual Warburg apparatus at 30.4°C. In most cases,  $O_2$ -uptake or acid production was measured. In the case of the former, the center well of the flasks contained alkali. In the latter case, the acid was determined by the liberation of  $CO_2$  from bicarbonate buffer. Any  $CO_2$  or other gas produced will be included in this measurement. The other components of the flask are given in the material accompanying the individual tables.

Sodium hexosediphosphate was prepared from the commercial barium salt and sodium pyruvate by carefully neutralizing a weighed amount of vacuum-distilled acid. Succinic acid was determined manometrically with succinic dehydrogenase (Krebs, 1937) or gravimetrically as the silver salt (Wood *et al.*, 1942).

## EXPERIMENTAL

*Anaerobic Activity of Extracts of Propionibacterium.* Extracts from *Propionibacterium pentosaceum* (49) metabolized several substrates both aerobically and anaerobically and thus denoted the presence of a large variety of enzymes. The properties of extracts from *P. pento-*

TABLE I  
*Activity of Extracts from Propionibacterium pentosaceum*

Substrate	Anaerobic		Aerobic	
	Extract from:		Extract from:	
	24-hour cells	40-hour cells	24-hour cells	40-hour cells
None	106	54	58	80
Glucose	233	38	99	93
Glucose+	206	352	—	—
Hexosediphosphate				
Pyruvate	199	627	44	83
Fumarate	147	454	53	210
Succinate	50	90	117	187
Glycerol	230	13	286	138
Lactate	116	39	178	317

Activity expressed as cu. mm.  $CO_2$  or  $O_2$ -uptake per hour per vessel. Each vessel contained 1.2 ml. enzyme (for preparation see Kalnitsky *et al.*, 1945) and, expressed as final concentration, 0.03 *M* substrate; 0.045 *M*  $NaHCO_3$ ; 0.05 *M*  $Na_2HPO_4$ — $KH_2PO_4$  buffer (pH 6.8) in a total volume of 2.0 ml. Atmosphere 10%  $CO_2$  in  $N_2$  or air.

*saceum* are influenced markedly by the age of the cells from which the extracts are prepared. A summary of the results of a typical anaerobic experiment is presented in Table I. Extracts were prepared from cells aged 24 and 40 hours for this experiment.

Anaerobically, the 24-hour extract has a considerable endogenous acid production, but the activity is increased by addition of glucose,

pyruvate, glycerol and slightly by fumarate. Hexosediphosphate (HDP) does not enhance the activity with glucose as the substrate. It has been shown that the glycolytic activity of cell-free extracts from yeast (Meyerhof, 1918), brain (Utter *et al.*, 1945) and bacteria (Utter, 1942) is increased by addition of HDP.

The 40-hour extract differs by exhibiting a lower endogenous acid production and a much higher rate on pyruvate and fumarate. On the other hand, glucose and glycerol no longer support acid production although addition of HDP to the former enables rapid glycolysis to occur.

In general, three points of difference can be noted between the two extracts: (a) higher activity on pyruvate and fumarate in the 40-hour extract; (b) necessity of HDP for glycolytic activity in the 40-hour extract; (c) cessation of activity upon glycerol in the 40-hour extract.

Although HDP is necessary for glycolysis with the 40-hour extract and is not necessary with the 24-hour extract, it does not necessarily follow that the two extracts possess different mechanisms of glycolysis. HDP may be necessary as a reservoir of organic phosphate during glycolysis if phosphatase activity is high or, conversely, if esterification reactions are slow (Utter *et al.*, 1945). It is interesting to note, however, that this particular organism was used by Wiggert and Werkman (1939) in the demonstration of a NaF-insensitive glycolysis. There was some evidence to indicate that phosphoglyceric acid was not involved as an intermediate of glycolysis in the NaF-insensitive cells.

The inability of the older extract to utilize glycerol is unexplained, but it is possible that the inactivity signalizes the disappearance of one or more enzymes present in the 24-hour extract. The ability of an extract to utilize glycerol should be of considerable aid in tracing the metabolic path of this substance.

Neither preparation produced appreciable quantities of acid from lactate or succinate.

*Oxidative Activity of Propionibacterium Extracts.* Extracts from *P. pentosaceum* are able to oxidize several substrates (Table I) as determined by an increase in  $O_2$ -uptake over the endogenous caused by addition of a substrate. Aerobically, the properties of the extracts are again affected by the age of the cells from which the extracts are prepared. The 24-hour extract oxidized glycerol and lactate rapidly, succinate and glucose slowly, pyruvate and fumarate not at all.

With an extract from older cells, the rate of oxidation on lactate and succinate is increased, and in addition, fumarate is now oxidized. Glycerol oxidation, however, is very slow and glucose oxidation has almost ceased. Pyruvate is unattacked as before.

The marked aerobic activity is somewhat surprising since it has generally been found impossible to obtain complete oxidative systems in preparations free from larger particles. The extract used in this experiment was almost water clear, and it is not probable that particles of any considerable size were present.

Both aerobic and anaerobic activities are affected somewhat similarly by cell age, strengthening the view that fundamental differences in enzymes (or coenzymes) exist between the two extracts. For example, the activity upon glycerol is much higher with the 24-hour extract both aerobically and anaerobically. In general, the activity upon pyruvate, lactate, fumarate and succinate increases with the 40-hour extract.

It has long been known that the enzymatic nature of bacterial cells is influenced by growth conditions (*cf.* review by Gale, 1943); most of these experiments have been carried out with intact cells and it has been impossible to weigh the role of permeability in such experiments. Woolridge *et al.* (1936) showed that the dehydrogenase activity of *Escherichia coli* suspensions is a function of age and that the highest activity is apparently obtained during the logarithmic phase of growth.

Likewise, it is clear from Table I that differences exist between extracts from cells of different ages. The source of these differences is not certain. At least two explanations are possible: (a) fundamental changes may occur in the enzyme or coenzyme structure of the cells with age, and these changes are reflected in the extracts; (b) changes may occur in the physical properties of the cells which affect the mechanical process of extract preparation and, hence, the resulting extract. For example, the age of the cells may affect the grinding, extraction or centrifugation.

In studies on the effect of pH of the growth medium on the enzymes of *E. coli* and *Micrococcus lysodeikticus*, Gale and Epps (1942) found that the enzymatic variation observed in the latter species could be shown either with intact or lysed cells. The authors thus concluded that the actual enzymatic nature of the cells had changed.

*Proteus Extracts.* Extracts obtained from cells of *Proteus morganii* (M21) grown on the usual medium exhibited oxidative activity but

did not produce acid or gas anaerobically (Table II). The preparation is able to oxidize the four-carbon dicarboxylic acids and also lactic acid, although pyruvic acid and glucose are not oxidized by this preparation.

In corresponding experiments not shown, the extracts were found to produce negligible amounts of  $\text{CO}_2$  from bicarbonate buffer in the presence of the same substrates listed in Table II.

TABLE II

*Aerobic Activity of a Preparation Obtained from Proteus morganii (M 21)*

Substrate:	None	Glucose	Pyruvate	Lactate	Succinate	Fumarate
Cu. mm. $\text{O}_2$	40	34	42	258	235	182

Each vessel contained: enzyme, 0.8 ml.; phosphate buffer, pH 6.8, 0.05 *M*; substrate, 0.03 *M*; total volume, 2.0 ml., plus 0.3 ml. 20% KOH in alkali well. Atmosphere, air. Duration, 1 hour.

*Extracts from Escherichia coli.* Extracts from *Escherichia coli* (E26) have been studied extensively in earlier reports (Kalnitsky and Werkman, 1943, 1944; Kalnitsky *et al.*, 1943; Utter and Werkman, 1941, 1942, and 1944), and considerable information is already available concerning the enzymes found in extracts from this strain. However, no general survey of the activity of such extracts has been presented as previous work has been taken up with a limited number of specific enzymes. It is interesting to find that growth conditions play an important role in determining the properties of the extracts as was the case with *Propionibacterium*.

In contrast to extracts of *Propionibacterium* and *Proteus*, extracts from *E. coli* have proved to be relatively inactive aerobically. Table III shows typical activity anaerobically on various substrates as indicated by gas production in bicarbonate buffer.

TABLE III

*Activity of Preparation Obtained from Escherichia coli*

Substrate:	None	Glucose	Pyruvate	Succinate	Fumarate	Lactate	Glycerol
Cu. mm. $\text{CO}_2$	39	710	230	48	206	23	10
1st hour							

Cells grown on glucose-yeast extract medium.

Each vessel contained enzyme, 0.8 ml.;  $\text{NaHCO}_3$ , 0.045 *M*; phosphate buffer, pH 6.8, 0.025 *M*; substrate, 0.03 *M*; total volume, 2.0 ml.; atmosphere, 10%  $\text{CO}_2$  in  $\text{N}_2$ .

In this experiment the cells were grown on the usual glucose-yeast extract medium. Acid is produced very rapidly from glucose, and fairly rapidly from pyruvate and fumarate. The extract is unable to ferment succinate, lactate, or glycerol under the conditions of the experiment.

It has been shown previously that pyruvate is converted to lactic, acetic and formic acids with this extract (Utter and Werkman, 1944) and it is probable that glucose is fermented to similar products, at least qualitatively. The nature of the fumarate fermentation has not been investigated, but Krebs (1937) has shown that fumarate undergoes a dismutation to succinate and  $\text{CO}_2$  with intact cells of this species.

*Effect of Growth Media on E. coli Extracts.* In addition to the glucose-yeast extract medium (termed A) usually employed, extracts have been prepared from cells grown on two other media: (B) a glucose medium similar to (A) in which peptone replaces yeast extract, and (C) a non-carbohydrate medium of beef extract, yeast extract, etc.

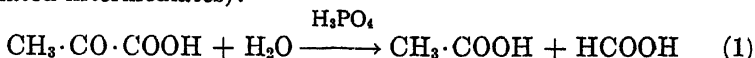
A comparison of the extracts from cells grown on various media is given in Table IV. Substitution of peptone for yeast extract has a considerable effect on the resulting extracts in that the activity is greatly reduced on glucose and disappears entirely with pyruvate as the substrate. Addition of hexosediphosphate aids only slightly with extract B. It is possible that the great diminution of activity observed here is due to a smaller store of the coenzymes of glycolysis in cells grown on the "poorer" peptone medium. The fact that the addition of boiled yeast extract as a source of coenzymes caused only a small increase in both extracts A and B (Utter, 1942) makes such an explanation unlikely.

The extract obtained from cells grown on a non-carbohydrate medium (C) is inactive on glucose, but the activity on pyruvate is extremely high. Little acid is produced from fumarate, but the extract can utilize gaseous hydrogen to reduce fumarate. Extract A is much less active in this respect.

Epps and Gale (1942) showed that the presence of glucose in the culture medium influenced the activity of cell suspensions of *E. coli* by depressing the deaminase activity and increasing the ability to ferment glucose. The latter finding is in general accord with the differences observed in Table V with *E. coli* extracts.

A comparison of extracts A and C with pyruvate as the substrate (Table V) reveals that the properties of this enzyme system in the two

extracts are quite dissimilar despite their common origin from the same strain of *E. coli*, and that both extracts have been shown to dissimilate pyruvate in the main by Reaction 1 (disregarding phosphorylated intermediates):



For example, differences between the extracts can be observed in

TABLE IV

*Variation in Extracts Obtained from E. coli Grown on Different Media*

Substrate	Cu. mm. CO <sub>2</sub> in 1 hour by Extracts from cells grown on		
	Medium A	Medium B	Medium C
Glucose (0.01 M)	603	117	—
Glucose + Hexosediphosphate	632	150	30
Pyruvate (0.02 M)	361	25	—
Pyruvate (0.015 M)	—	—	301
Pyruvate (0.03 M)	—	—	530
Succinate (0.03 M)	48	—	22
Fumarate (0.03 M)	206	—	65
	Cu. mm. H <sub>2</sub> taken up in 1 hour		
Fumarate (0.03 M)	-74	—	-210

*Preparation A* was obtained from cells grown in usual glucose-yeast extract medium.

*Preparation B* was obtained from cells grown in usual medium except that the yeast extract was replaced by peptone.

*Preparation C* was obtained from cells grown in the following medium: Beef extract, 0.3%; yeast extract, 0.3%; peptone, 0.3%; NaCl, 0.2%; tap water, 10%; distilled water to volume; medium aerated during growth.

For experiments measuring CO<sub>2</sub> evolution: vessels contained enzyme, 0.8 ml.; NaHCO<sub>3</sub>, 0.045 M; substrated in indicated concentrations; HDP, 0.005 M; total volume, 2.0 ml.; atmosphere, 10% CO<sub>2</sub> in N<sub>2</sub>.

For experiments measuring H<sub>2</sub> uptake: vessels contained enzyme, 0.6 ml.; substrate in indicated concentration; atmosphere, H<sub>2</sub>; total volume, 2.0 ml.

activity, pH range, phosphate requirements and susceptibility to inhibitors. To consider these differences in more detail: the activity of extract C for the first 15 minutes is about three times that of extract A. The difference in the pH range is most apparent in the higher pH range. Preparation A retains 100% of its maximal activity at pH 7.5, whereas preparation C retains but 21% of the maximal value. Preparation A has a phosphate optimum at 0.05 M, whereas preparation C exhibits optimal activity at 0.018 M. In part (d) of Table V, it can be

seen that preparation C is much more sensitive to inhibition by KCN than is A. The preparations differ in one additional respect, the ability to form succinate from pyruvate. Kalnitsky, Wood and Werkman (1943) showed that succinate is formed from pyruvate by preparations similar to C, and by the aid of isotopic carbon have shown that  $\text{CO}_2$  is fixed in the succinate.

An interesting question is opened by the different properties of the two extracts. Since the nutrients available for cell growth vary in the

TABLE V  
*Comparison Between Two Pyruvate Enzyme Systems Obtained from E. coli*

Conditions	Preparation A	Preparation C
(a) Rate of reaction:		
Cu. mm. $\text{CO}_2$ per first 15 min.		
Conc. of pyruvate: 0.015 M	—	256
0.02 M	84	—
0.03 M	—	318
(b) pH effects: per cent of maximum activity retained at pH 7.5	100	20
(c) Optimum phosphate concentration (M)	0.05	0.018
(d) Per cent inhibition produced by concentration of KCN: 0.0025 M	7.0	—
0.005 M	—	95.0
0.05 M	12.0	—
0.01 M	21.0	—
(e) Formation of succinate in mm. per 100 mm. of pyruvate fermented	trace	12-14

Tested as described in Table V for  $\text{CO}_2$  evolution with indicated variations. (In (b), pH actually varied in the following manner: Enzyme preparation was originally extracted with water and phosphate buffer of appropriate pH was placed in vessels; atmosphere,  $\text{N}_2$ ; activity determined by analyzing for amount of pyruvate fermented.)

two cases, it is possible that the protein composition of the resulting cells are altered. In the same way it is possible that a particular enzyme system may be constructed from different components (amino acids, etc.) when the medium is varied, with resulting differences in properties. A study of amino acids and other components of purified enzymes may be necessary to decide this point.

The differences in succinate production observed in the different *E. coli* extracts can also be demonstrated by using resting cells grown on different media. Krebs (1937) showed that *E. coli* forms large

amounts of succinic acid from galactose or pyruvate but very little from glucose. The effect of the growth medium has been more thoroughly investigated and the results are shown in Table VI. Cells grown on an agar-beef extract medium as described by Krebs formed the largest amounts of succinate. Cells grown in a liquid-beef extract medium formed fairly large amounts of succinate from pyruvate. Aeration had little effect. Addition of glucose to the medium greatly diminished the ability to form succinate. When the medium containing glucose was kept alkaline at all times, the ability of the resulting cells to form succinate from pyruvate was not increased.

TABLE VI

*Variation in Amounts of Succinic Acid Produced by E. coli Grown in Various Media*

Medium	mM Succinic acid per 100 mM pyruvic acid fermented
1. Agar-beef extract	19.2
2. Liquid beef extract	12.6
3. Liquid beef extract plus aeration	11.5
4. No. 2 plus glucose	8.9
5. No. 2 plus glucose kept alkaline	3.4
6. Glucose	4.2
Medium 1: Beef extract, 1.0%; yeast extract, 0.5%; peptone, 1.0%; NaCl, 0.5%; agar, 3.0%; tap H <sub>2</sub> O, 10.0%.	
Medium 2: Beef extract, 1.0%; peptone, 0.4%; yeast extract, 0.5%; NaCl, 0.5%; tap H <sub>2</sub> O, 10.0%.	
Medium 3: Same as Medium 2 plus aeration.	
Medium 4: Same as Medium 2 plus 1% glucose.	
Medium 5: Same as Medium 2 plus 1% glucose and aeration.	
Medium 6: Glucose, 1.0%; peptone, 0.4%; K <sub>2</sub> HPO <sub>4</sub> , 0.8%; tap H <sub>2</sub> O, 10.0%.	

*General Applicability of the Glass-grinding Method.* In addition to the preparations from *Propionibacterium*, *Proteus* and *Escherichia* already described, active extracts from the following species have been obtained by application of the glass-grinding technique in this laboratory.

*Aerobacter.* Extracts from *Aerobacter indologenes* brought about the reduction of methylene blue by galactose, formate, fumarate, lactate, xylose, dihydroxyacetone and pyruvate (Wiggert *et al.*, 1940). Preparations from *A. aerogenes* were shown by Silverman and Werkman (1941) to form large quantities of acetylmethylcarbinol and CO<sub>2</sub> from pyruvate.

*Staphylococcus aureus.* An extract from this organism causes a dis-



mutation of pyruvate to lactic acid, acetic acid and  $\text{CO}_2$  (Krampitz and Werkman, unpublished).

*Micrococcus lysodeikticus*. Extracts from this organism oxidize fumarate and malate slowly, but the rate is increased by addition of methylene blue as a carrier. The same preparations are unable to oxidize pyruvate, acetate, succinate or glucose (Utter and Werkman, unpublished).

*Thiobacillus thio-oxidans*. This extract shows endogenous uptake of  $\text{CO}_2$  and  $\text{O}_2$  and the presence of sulfur increases the rate (Subtelny and Werkman, unpublished). The autotrophic nature of this organism makes this extract particularly interesting.

*Clostridium butylicum*. An extract from this species produced acid anaerobically from fumarate, lactate, pyruvate and oxalacetate (Brown *et al.*, 1944).

*Mycotorula lipolyticum*. This organism yielded an active lipase preparation which liberated butyric acid from butter fat (Kalnitsky and Werkman, unpublished).

*Saccharomyces cerevisiae*. An active extract was obtained from dried brewer's yeast by moistening with a little water and grinding with glass. The carboxylase activity of this preparation was about one-third that of Lebedev juice from the same yeast.

The method has been unsuccessful only with *Lactobacillus arabinosus* and *L. casei*, the extracts being only slightly active in most cases on the substrates of Table I.

## DISCUSSION

It is evident that the glass-grinding method can be applied to a wide range of microorganisms. With a few exceptions the enzymes thus far demonstrated in extracts have been concerned with carbohydrate metabolism. However, few attempts have been made to demonstrate enzymes involved in other metabolic areas.

As has been pointed out previously (Kalnitsky *et al.*, 1945), the glass-grinding method possesses the advantage that the equipment required is simple and inexpensive and involves only glass surfaces. The method has a disadvantage in that a considerable fraction of the bacterial cells are not disrupted by the procedure, necessitating their removal in most cases by centrifugation. All large cell fragments will also be removed by this procedure. In practice, therefore, the pro-

cedure has been limited to enzymes that are relatively soluble. There is no reason, however, that careful differential centrifugation cannot be employed to obtain fractions containing particles of desired sizes, thus extending the procedure to other enzymes.

### SUMMARY

1. The glass-grinding method for preparing cell-free extracts has been applied successfully to 8 *genera* of bacteria and 2 *genera* of yeasts.

2. Extracts from *Propionibacterium*, *Escherichia* and *Proteus* have been examined for activity on various substrates both aerobically and anaerobically. Extracts from the first two *genera* vary markedly with the growth conditions of the cells from which the extracts are prepared. With *Propionibacterium*, the age of the cells has a considerable effect on the nature and the activity of the enzymes of the extracts. With *Escherichia* extracts, the composition of the medium is shown to affect the properties of the enzyme systems present.

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## On the Mechanism of Enzyme Action.

### Part 27. The Action of Certain Wood-Destroying Fungi on Glucose, Xylose, Raffinose and Cellulose<sup>1</sup>

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#### INTRODUCTION

In recent years the biochemistry and enzymology of *Fusaria* have received considerable attention. These molds are characterized by the absence of significant amounts of organic phosphorus donors, by the presence of a powerful and diversified dehydrogenating system and by their ability to completely ferment hexoses as well as pentoses via pyruvic acid, bypassing the phosphoglyceric acid step. Moreover, they are able to reduce nitrates via nitrite to hydroxylamine, to utilize potassium cyanide or elementary sulfur, and to synthesize a variety of pigments which can serve as mediators between oxidation and assimilation. Some of these molds also possess a high thiamine content, abundantly convert carbohydrates into fats, and the action of certain enzymes present in them is somewhat inhibited by *p*-amino-benzoic acid (1).

In the light of the versatility of the *Fusaria* and their enzymatic unsaturation, which prevent a close integration of their action, it was deemed advisable to attempt a renewed approach to the problem of the mechanism of wood decay. Comparatively few unequivocal and tangible facts (2) are available in this field. The functional description of certain enzymes present in wood-attacking fungi appears to be somewhat haphazard. This, in turn, seems to be caused by a lack of

<sup>1</sup> For a preliminary report see: *Nature* 157, 335 (1946). Presented before the Division of Sugar Chemistry and Technology of the Am. Chem. Soc., Atlantic City, N. J., April, 1946.

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<sup>3</sup> Communication No. 44.

systematic deployment and to be due to the uncertainty of, or difficulty in, making a clear-cut subdivision of wood-destroying fungi. As a matter of fact, Bose (3) claims that, of the wood-rotting organisms, only about two dozen have been studied with regard to their enzyme activity.

Wehmer (4) aptly called the wood-destroying fungi the pioneers in a process which serves to reintroduce the gigantic wood waste into the basic cycle of utilization. Three general types of rot have been postulated in the breakdown of wood by fungi and, in the third form, again two sub-groups have been distinguished (5), *viz.*, those which destroy cellulose in preference to lignin and those which attack cellulose *and* lignin. Waksman (6) states that the mechanism of cellulose degradation by bacteria and fungi is not fully understood because intermediary substances have been but rarely isolated. Accordingly, it was considered desirable to attempt a preliminary attack on the problem in its fundamentals and first to deploy the course of oxidation of glucose, xylose, raffinose and cellulose by certain *Merulia* and by *Fomes annosus*.

#### EXPERIMENTAL

The molds studied in this investigation were the following: *Merulius niveus* (Meni), *Merulius tremellosus* (Metre), *Merulius confluens* (Meco) and *Fomes annosus* (Foman). They are commonly known as wood-rotting fungi. The cultures were obtained through the courtesy of Dr. William J. Robbins of the New York Botanical Gardens and were, according to information received from Dr. F. Kavanagh, previously maintained on a medium consisting of malt extract, Difco. 20.00 g., agar 15.00 g. and water to one liter.

All the chemicals used were of tested purity grade. The cellulose was chemically prepared Whatman filter paper No. 2, made in England.

#### *Establishment of Growth Conditions*

*Growth Factors.* In exploratory experiments the effect of *p*-amino-benzoic acid (7), nicotinic acid, inositol, desthiobiotin and thiamine (8) on these molds was investigated. It was established that it was necessary to add thiamine (9) to the chosen medium to ensure a growth satisfactory for the study of enzyme action. Metals, such as zinc, copper, iron or manganese, when used in traces, did not show any measurable effect on the growth.

*Nitrogen Sources.* Among the nitrogen sources used, peptone, urea and asparagine proved the most utilizable for these molds and supported an abundant growth. Potassium nitrate, sodium nitrate, ammonium nitrate, ammonium sulfate and ammonium phosphate, containing inorganic nitrogen, were, contrary to their utilization by *Fusaria* (1), poor nitrogen sources. The molds grew very slowly and with a scanty mycelium.

*Carbon Sources.* Glucose and xylose proved excellent carbon sources for all four organisms. Raffinose, cellulose, in the form of filter paper, and inositol supported growth of all four organisms.

These molds were able to attack spruce wood and red wood when ammonium sulfate or ammonium phosphate was used as a nitrogen source. Hemlock and spruce wood were also attacked more successfully when the nitrogen utilized was derived from peptone. Pine and cedar wood were not visibly attacked.

#### *Experimental Conditions and Microbiological Methods*

The following semi-synthetic medium was finally adopted for stock cultures, preparations of inoculum, qualitative and quantitative experiments:

1.50 g. Peptone
1.50 g. $\text{KH}_2\text{PO}_4$
0.50 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
30.00 g. Glucose U.S.P. or
Raffinose or
20.00 g. Xylose or about
24.00 g. Cellulose
2.00 mg. Thiamine hydrochloride
Tap water to 1000 ml.

Stock cultures of these organisms were also maintained in which peptone was replaced by an equal amount of asparagine, urea or potassium nitrate. All four organisms were also cultivated in a peptone medium containing xylose or raffinose.

The molds were subcultured in the above mentioned media every month. Inoculations were made with a mycelial spore suspension prepared by growing the molds on a solid medium contained in a 125 ml. Erlenmeyer flask. The medium for this purpose was the same as above, supplemented by Difco agar for solidification. After growth had progressed for a period of about two weeks, 50 ml. of sterile, distilled water was added to each flask containing the solid medium and spore-mycelial suspensions prepared by scraping the surface of the plate. Usually 1 ml.

of the mycelial-spore suspension was added for each 50 ml. of medium. Occasionally inoculations were made with bits of mycelium as in qualitative experiments.

Stock cultures were maintained, growth experiments and quantitative experiments were carried out in 125 ml. Pyrex Erlenmeyer flasks, using 50 ml. of media for aerobic experiments and 100 ml. for experiments carried out in a nitrogen atmosphere. Experiments with cellulose and wood were performed in 500 ml. capacity Fernbach flasks with 100 ml. of medium; those involving identification and isolation of metabolic products in Fernbach flasks of 3 l. capacity, containing one liter of medium.

Flasks, in which experiments under aerobic conditions were carried out, were plugged with non-absorbent cotton, while in incubating experiments conducted anaerobically, the flasks were plugged with a two-hole rubber stopper fitted with two glass tubes bent at right angles, connected by means of rubber tubing and flushed with nitrogen taken from a tank and subjected to passage through a mercuric chloride solution.

The media were sterilized, according to requirements, at 20 lbs. pressure for 20 minutes or more, depending on the depth of the medium in the flask. Substances which would have been materially affected by pressure sterilization and pH were added with sterile technique in a solution of sterile distilled water.

The molds investigated were incubated at 28°C. in the dark.

### *Analytical Methods*

The contents of at least three flasks were taken together for analyses made in duplicate. Uninoculated blanks were analyzed simultaneously.

*Carbohydrates.* Glucose and raffinose were determined quantitatively by means of a polarimeter. As the peptone used had a slight negative rotation the glucose left unfermented was frequently determined by the copper reduction method (10). In no case were measurable deviations found. Xylose, having a low specific rotation, was analyzed by the copper reduction method of Lehmann-Maquenne-Schoorl (11).

*Ethyl Alcohol* was determined by the oxidation method of Janke and Kropacsy (12). In our procedure, carbohydrates were removed by precipitation with  $\text{Ca}(\text{OH})_2$ - $\text{CuSO}_4$  (13). Possible interference of aldehydic compounds was prevented by addition of a small amount of silver sulphate before distilling the ethanol.

Acetic acid was removed from the acidified media by steam distillation and an aliquot titrated with 0.01 *N* NaOH to the phenolphthalein end point.

*Phosphate* was determined by the method of Fiske and Subbarow (14).

Other special methods will be discussed in the course of the presentation of results.

*Mycelial Weights* were determined by filtering the mycelia by means of tared porous alundum crucibles, washed with several portions of water (total volume 150 ml.), dried overnight in an oven at 60°C. and weighed.

All pH determinations were made electrometrically, using a glass electrode. For adjustment of the pH KOH or  $\text{H}_3\text{PO}_4$  was used.

### *Detection and Identification of Metabolic Products of Glucose Dissimilation*

*Procedure.* As previously mentioned, qualitative experiments were carried out by fermenting one liter of medium in three liter Fernbach flasks. When it was noticed

that a substantial change had taken place, the medium was separated from the synthesized mycelium by filtration, the pH and glucose determined in an aliquot, and the remainder neutralized and immediately fractionated, using a 2 foot fractionating column. The collected distillate was brought to a definite volume, usually 250 ml., or further concentrated and analyzed for volatile neutral products. The residue was analyzed for nonvolatile acids and, in case of scarcity, a portion utilized for determination of volatile acids. These acids, in general, were detected and analyzed in the untreated portion of the filtrate.

### *Volatile Neutral Products*

*Alcohols.* An aliquot of the distillate was oxidized with dichromate and sulfuric acid for an hour at 40°C. on a water bath. The mixture was steam distilled and, in one part of the distillate, the distribution constant (15) determined. The remaining distillate was subjected to Duclaux distillation (16).<sup>\*</sup> The constants obtained pointed to the presence of ethyl alcohol and simultaneously indicated the absence of methyl alcohol or higher alcohols. The alcohols obtained from Meni, Metre and Foman were identified on the 13th day of growth, and from Meco on the 20th day of growth.

The alcohol produced by the latter mold was further identified on 23rd day of growth by a derivative. Two liters of medium were fractionated and concentrated to small volume and *p*-nitrobenzoylchloride added. The crude derivative melted at 53–55°C. After three recrystallizations from dilute alcohol the melting point was 56–57°C.; N found 7.18%; calc. 7.44%.

*Aldehydes.* Among the aldehydes isolated without interception was acetaldehyde. Tests for formaldehyde with chromotropic acid (18) were negative. Acetaldehyde was qualitatively detected with the piperidine nitroprusside test (19) on the 11th and 17th day in the first 30 ml. of the fractional distillate. The test proved very strong in the case of Meco, weaker with Meri and Metre and only a trace of coloration was observed with Foman.

The acetaldehyde was therefore best identified with Meco. On the 14th day of growth 1 liter of medium was fractionated as above and to the first 100 ml. of distillate 2,4-dinitrophenylhydrazine in 2 *N* HCl was added. The voluminous flocculent precipitate was collected and amounted to 32 mg. of the 2,4-dinitrophenylhydrazone, M.P. 147°C.

<sup>\*</sup> Whittier and Sherman (17) make the following statement: "The Duclaux method gave very consistent results; it is difficult to understand why investigators so frequently attempt to modify it."



(20). In the case of Meri and Metre the 2,4-dinitrophenylhydrazine reagent only gave rise to a slight opalescence.

*Other Compounds.* When, however, the same reagent was added to the first distillate obtained with Foman, the deposition of an abundant red precipitate was observed. The precipitate was allowed to stand over night, filtered and dried *in vacuo*. Forty-five hundredths mg. of crude 2,4-dinitrophenylhydrazone was obtained from the fractional distillate of the medium, having a melting point of 198°C. This compound is formed when Foman is grown on either a solid or a liquid medium and isolated after steam distillation and ether extraction. In the first case the medium, together with the mycelium, was subjected to the distillation and then extracted. In the second case, only the mycelium was steam distilled and the medium was directly extracted. The 2,4-dinitrophenylhydrazone has a M.P. of 208°C. after repeated recrystallizations from alcohol.

Two microanalyses of products of two independent series of experiments gave the following values:

C = 59.86%	H = 4.75%	N = 13.07%
C = 60.03%	H = 4.65%	N = 13.75%

Considering the analyses, a possible overall formula for the base would be  $C_{14}H_{14}O_2$ . The pure base has a low melting point and is very soluble in ether, soluble in alcohol and insoluble in water. The isolation of such a compound could indicate that, in addition to the enzymes of oxidation, an enzyme perhaps capable of bringing about a true C-C linkage may also be present in Foman.

*Volatile Acids.* The volatile acids were identified by steam distilling a portion of the medium strongly acidified with  $H_2SO_4$ . In the distillate the partition constant was determined and the remainder was subjected to fractionation. These determinations served to establish the presence of and identify acetic acid which gave the highest yields in the case of Meco. Overall data on the acetic acid accumulation are presented below:

Meri	Metre,	Meco	Foman
Traces	6.8 mg./100 ml.	24 mg./100 ml.	5.2 mg./100 ml.

The determination was run on the 17th day.

In Table I a record of an experiment completed on the 30th day is presented:

TABLE I

	pH	Glucose <sup>2,4</sup> left	Alcohol <sup>1</sup>	Mycelium wt. <sup>4</sup>	Acetic Acid <sup>1</sup>
Meni	3.7	1.90	8.8	291.7	none
Metre	3.2	1.53	6.9	182.7	2.42
Meco	—	1.53	2.80	68.5	23.4
Foman	3.5	2.10	8.1	26.8	—
Blank	4.95	3.20	—	—	—

<sup>1</sup> Results expressed in mg./100 ml.<sup>4</sup> Glucose expressed in g./100 ml.

The accumulated acetic acid amounts, in the case of Meco, to 1.49% of the glucose which has disappeared.

*Nonvolatile Acids.* The residue from the distillation of the neutral products was concentrated to small volume *in vacuo*, evaporated to a syrup on the steam bath, mixed with anhydrous Na<sub>2</sub>SO<sub>4</sub> and extracted with ether in a Soxhlet apparatus for 16 hours. The ether was again evaporated, the residue taken up in water and brought to a definite volume. Attempts to detect and identify pyruvic, citric, tartaric and oxalic acids on the 11th, 17th and 30th day of degradation were futile. The specific test for the identification of 1,2-dicarboxylic acids with freshly sublimed resorcinol and conc. sulfuric acid was, however, positive, indicating the presence of succinic acid.

### Quantitative Experiments

To establish quantitative relations in the carbohydrate degradation caused by the four molds, the influence of the initial pH was investigated in the peptone-glucose medium mentioned above. From the data recorded in Table II it can readily be seen that the wood-rotting molds under investigation have a range of growth reaching approximately from pH 3 to pH 8, the optimum pH for all the organisms being on the acid side as could be expected for such microorganisms. An initial pH of 5 was, therefore, chosen in all quantitative experiments presented.

### Glucose Fermentation

In the glucose dissimilation the ethyl alcohol accumulated is in the ratio of 1:2. For one mole of glucose utilized, two moles of ethyl alcohol are obtained. (See Table III.) Larger accumulations of alcohol were especially noticeable with Meco. On cultivating this fungus on

TABLE II  
*Effect of Initial pH on Growth in a Peptone-Glucose Medium*

Mete													Foman														
Mete													Foman														
Day	pH	Mete											Foman														
		0	1.8	2.31	2.78	2.98	4.05	4.48	5.05	5.70	6.60	7.10	7.30	7.90	1.8	2.31	2.78	2.98	4.05	4.48	5.05	5.70	6.60	7.10	7.30	7.90	
0	Glucose, g.	2.77	2.78	2.78	2.78	2.78	2.77	2.75	2.74	2.74	2.74	2.74	3.18	3.20	3.00	2.78	2.78	2.78	2.78	2.77	2.75	2.74	2.74	2.74	3.18	3.20	3.00
15	pH	2.77	2.77	2.77	2.77	2.77	2.71	3.6	3.6	3.9	4.2	4.35	4.38	4.38	4.7	2.80	2.77	2.70	2.47	2.20	2.19	2.38	2.43	2.26	3.20	3.40	3.00
	Glucose, g.	2.77	2.77	2.77	2.77	2.77	2.71	1.52	1.86	1.85	1.72	2.62	2.64	2.80	2.80	2.80	2.77	2.70	2.47	2.20	2.19	2.38	2.43	2.26	3.20	3.40	3.00
30	Myccelium, mg.	none	none	36	328	343	349	350	297	266	258	262	262	262	262	262	12	217	234	326	277	270	23	6	none	none	none
	pH	2.0	2.35	3.44	3.60	3.60	3.60	3.45	4.70	4.62	4.52	4.55	4.55	4.55	4.55	4.55	2.0	2.35	2.90	3.50	3.60	3.30	4.10	1.40	3.90	—	—
	Glucose, g.	—	—	—	2.17	1.96	0.8	1.67	1.43	1.65	2.32	2.50	2.50	2.50	2.50	2.50	—	2.53	2.04	2.29	1.94	2.41	2.50	1.97	2.60	—	—
	Myccelium, mg.	none	none	413	428	109	453	308	359	389	387	363	363	363	363	363	none	35	359	396	508	413	162	307	126	none	none
Mete													Mete														
Mete													Mete														
Day	pH	Mete											Mete														
		0	1.8	2.31	2.78	2.98	4.05	4.48	5.05	5.70	6.60	7.10	7.30	7.90	1.8	2.31	2.78	2.98	4.05	4.48	5.05	5.70	6.60	7.10	7.30	7.90	
0	Glucose, g.	2.77	2.78	2.78	2.78	2.78	2.77	2.75	2.74	2.74	2.74	2.74	3.18	3.20	3.00	2.78	2.78	2.78	2.78	2.77	2.75	2.74	2.74	2.74	3.18	3.20	3.00
15	pH	2.77	2.77	2.77	2.77	2.77	2.71	3.6	3.6	3.9	4.2	4.35	4.38	4.38	4.7	2.80	2.77	2.70	2.47	2.20	2.19	2.38	2.43	2.26	3.20	3.40	3.00
	Glucose, g.	2.77	2.77	2.77	2.77	2.77	2.71	1.52	1.86	1.85	1.72	2.62	2.64	2.80	2.80	2.80	2.77	2.70	2.47	2.20	2.19	2.38	2.43	2.26	3.20	3.40	3.00
30	Myccelium, mg.	none	none	36	328	343	349	350	297	266	258	262	262	262	262	262	12	217	234	326	277	270	23	6	none	none	none
	pH	2.0	2.35	3.44	3.60	3.60	3.60	3.45	4.70	4.62	4.52	4.55	4.55	4.55	4.55	4.55	2.0	2.35	2.90	3.50	3.60	3.30	4.10	1.40	3.90	—	—
	Glucose, g.	—	—	—	2.17	1.96	0.8	1.67	1.43	1.65	2.32	2.50	2.50	2.50	2.50	2.50	—	2.53	2.04	2.29	1.94	2.41	2.50	1.97	2.60	—	—
	Myccelium, mg.	none	none	413	428	109	453	308	359	389	387	363	363	363	363	363	none	35	359	396	508	413	162	307	126	none	none

Results expressed in g. or mg. per 100 ml. of medium.

TABLE III

*Results of a Representative Glucose Fermentation*

Day	Meni				Metre				Meco				Foman			
	Glucose	Alcohol	Mycelium	pH	Glucose	Alcohol	Mycelium	pH	Glucose	Alcohol	Mycelium	pH	Glucose	Alcohol	Mycelium	pH
	g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.	
0	2.83	—	—	5.0	2.83	—	—	5.0	2.83	—	—	5.0	2.83	—	—	5.0
5	2.77	6	10	5.03	2.59	78	27	4.90	2.64	63	26	4.90	2.73	6	14	5.08
8	2.65	32	48	4.92	2.19	163	137	4.23	2.35	134	132	4.22	2.70	21	35	4.87
11	2.61	101	160	4.54	2.34	133	164	4.08	2.13	162	205	3.93	2.67	38	113	3.90
13	2.36	107	250	4.30	2.31	46	281	3.56	2.01	135	281	3.72	2.38	38	265	3.72
15	2.26	117	274	4.23	2.26	26	331	3.90	2.13	68	306	3.90	2.28	8	265	3.60
17	1.98	118	309	4.08	2.09	24	371	3.68	1.82	80	386	3.85	1.98	10	312	3.62
20	1.84	57	402	4.02	2.10	15	374	3.70	1.89	8	461	3.90	1.97	none	349	3.72
24	1.58	none	441	4.07	1.84	7	432	3.70	1.57	8	388	3.80	1.43	none	353	3.70
28	1.22	none	492	3.95	1.85	6	410	3.69	1.53	7	401	3.86	1.32	none	397	3.68

Results expressed in g. or mg. per 100 ml. of medium.

the glucose-peptone medium the mycelium developed partly submerged, bringing about significantly diminished losses of ethyl alcohol due to dehydrogenation occurring in the course of the degradation of glucose. When a culture that had been subcultured was compared with one which did not undergo many subcultivations, it presented unaltered morphological characteristics. Meri, Metre and Foman, on the other hand, seem to show that the alcohol formed is rapidly dehydrogenated. This is readily seen with Foman, in which case the amounts of alcohol actually accumulated are very small.

The dehydrogenation of the alcohol was further evidenced in experiments (Table IV) in which alcohol was the sole carbon source and in experiments under nitrogen which will be described later.

TABLE IV

*Dehydrogenation of Ethanol After 12 Days by Meni, Metre, Meco and Foman*

	Ethanol present	Ethanol utilized	Mycelial weight
Blank	139.69	0.0	0.0
Meni	60.7	79.0	73.0
Metre	85.2	54.0	42.0
Meco	113.5	26.0	8.0
Foman	55.9	83.7	34.0

Results expressed in mg./100 ml.

*Xylose Fermentation*

From Table V, recording data of a xylose oxidation, it can be seen that Meni gave rise only to traces of alcohol. The pH trend in the course of the xylose dissimilation by this mold was somewhat unusual, *viz.*, the pH followed the usual course of decrease only after an initial increase, thus enabling a faster degradation. With Metre and Meco the accumulation of alcohol during the degradation of xylose was comparable to that obtained from glucose. With Foman, again no alcohol was found to have been accumulated, indicating that the rate of dehydrogenation of alcohol practically equals the rate of formation.

TABLE V  
*Fermentation of Xylose*

Day	Meni				Metre				Meco				Foman			
	Xylose	Alcohol	Mycelium	pH	Xylose	Alcohol	Mycelium	pH	Xylose	Alcohol	Mycelium	pH	Xylose	Alcohol	Mycelium	pH
	g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.	
0	2.08	—	—	4.80	2.08	—	—	4.80	2.08	—	—	4.80	2.08	—	—	4.80
5	1.97	7	5	4.82	1.82	55	30	4.50	1.91	23	17	4.70	2.06	none	0	5.10
7	1.98	7	7	4.94	1.68	81	92	4.18	1.61	81	81	4.30	2.00	none	10	5.2
9	1.99	3	10	5.12	1.46	130	143	4.10	1.62	90	126	4.18	1.95	none	33	5.05
11	1.97	none	81	5.18	1.31	153	199	4.08	1.37	108	169	4.12	1.96	none	44	4.80
13	1.71	none	153	4.45	1.18	144	256	4.0	1.20	113	210	4.05	1.92	none	70	4.82
15	1.68	none	169	4.22	1.09	81	274	3.9	1.02	132	259	4.0	1.85	none	66	4.82
17	1.27	none	238	4.30	0.97	71	346	3.9	1.04	77	336	3.85	1.71	none	142	4.35
25	0.19	none	365	4.02	0.66	none	459	3.6	0.74	none	375	3.6	1.72	none	151	4.08

Values expressed in g. or mg. per 100 ml. of medium.

Although no carbon balance was made as in the glucose fermentations, it appears probable that the xylose fermentation satisfies the ratio 1:1, *i.e.*, one mole of xylose giving rise to one mole of alcohol as in *Fusaria* fermentations.

*Raffinose Fermentation*

Meni attacked Raffinose successfully in comparison with glucose (see Tables VIa and VIb). The trisaccharide was more rapidly utilized and, despite this rapid disappearance, the amount of alcohol accumulated was lower than in the parallel experiment with glucose. Moreover, the growth expressed by mycelial weights was lower than in the glucose experiments. This is in agreement with the higher rate of

TABLE VI A  
*Fermentation of Raffinose*

Day	Meni				Metre				Meco				Foman			
	Raffinose	Alcohol	Myceium	pH	Raffinose	Alcohol	Myceium	pH	Raffinose	Alcohol	Myceium	pH	Raffinose	Alcohol	Myceium	pH
	g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.	
0	3.07	—	—	5.30	3.07	—	—	5.30	3.07	—	—	5.30	3.07	—	—	5.30
7	2.91	8	19	5.26	3.00	3	16	5.02	3.06	12	17	5.10	3.05	trace	19	5.2
9	2.67	15	66	4.92	3.09	none	32	4.38	3.09	trace	18	5.00	3.06	none	42	4.3
11	2.37	27	140	4.68	3.07	none	43	4.20	3.11	none	27	5.20	3.09	none	71	4.5
14	2.32	66	266	4.28	3.08	none	61	4.20	3.12	none	25	5.56	3.00	none	155	4.2
17	1.79	22	321	—	3.10	none	73	4.68	3.07	none	46	—	2.90	none	198	4.06
21	1.56	10	380	4.12	3.05	none	93	4.64	3.06	none	71	4.75	2.77	none	237	3.95

Results expressed in g. or mg. per 100 ml. of medium.

fermentation of trehalose (21) as compared with that of glucose by *Fusarium lini* B.

Metre and Meco proved incapable of fermenting raffinose to any measurable degree. Foman after a weak initial growth did utilize raffinose to a small extent.

### Cellulose Fermentation

No intermediate products and only traces of alcohol were found to accumulate in the first days of aerobic cellulose utilization by these

TABLE VI B  
*Fermentation of Glucose*

Day	Meni				Metre				Meco				Foman			
	Glucose	Alcohol	Myceium	pH	Glucose	Alcohol	Myceium	pH	Glucose	Alcohol	Myceium	pH	Glucose	Alcohol	Myceium	pH
	g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.		g.	mg.	mg.	
0	2.84	—	—	5.07	2.84	—	—	5.07	2.84	—	—	5.07	2.84	—	—	5.07
7	2.76	30	28	5.00	2.66	73	60	4.77	2.68	66	22	4.68	2.81	11	15	4.95
9	2.66	59	92	4.55	2.60	92	68	4.40	2.60	107	33	4.54	2.78	24	37	4.90
11	2.39	97	251	4.43	2.64	100	120	4.26	2.36	200	71	4.24	2.75	31	91	4.38
14	2.26	135	336	4.35	2.55	48	237	3.94	1.98	367	89	4.10	2.64	40	177	3.83
17	2.12	138	367	4.19	2.55	28	285	3.59	1.27	694	137	3.94	2.55	22	255	3.80
21	1.84	14	515	4.12	2.26	26	357	3.68	0.34	992	133	3.95	2.13	21	330	3.62

Results expressed in g. or mg. per 100 ml. of medium.

organisms, although good growth was apparent. Analyses were performed on the 8th, 13th, 17th, 21st, 24th and 41st days after inoculation. This observation is, no doubt, due to the fact that the rate of dehydrogenation of the alcohol formed equals or exceeds the rate of cellulose degradation limited by its rate of diffusion.

### *Anaerobic Fermentation of Glucose, Xylose, Raffinose and Cellulose*

The fermentation of *glucose, xylose, raffinose and cellulose* was studied anaerobically under nitrogen to establish the effect of the absence of a hydrogen acceptor on the degradation of these substrates and on the possible accumulation of intermediates.

TABLE VII

#### *Anaerobic Fermentation of Glucose, Xylose, Raffinose and Cellulose*

Day		Meni				Metre				Meco				Foman			
		Glucose	Xylose	Raffinose	Cellulose	Glucose	Xylose	Raffinose	Cellulose	Glucose	Xylose	Raffinose	Cellulose	Glucose	Xylose	Raffinose	Cellulose
0	pH	4.78	4.77	5.25	5.20	4.78	4.77	5.25	5.20	4.78	4.77	5.25	5.20	4.78	4.77	5.25	5.20
	Substrate, g.	2.83	1.97	2.85		2.83	1.97	2.85		2.83	1.97	2.85		2.83	1.97	2.85	
40	Substrate, g.	2.41	1.81	2.07		1.68	1.24	2.40		2.50	1.92	2.83		2.50	1.92	2.70	
	Alcohol, mg.	175	15	107	281	506	187	64	119	181	9	6	4	123	10	31	3
	Acetic Acid, mg.	none	—	—	none	10.2	—	—	trace	18	—	—	—	trace	—	—	trace
	Mycelium, mg.	19	5	77	89	25	33	—	—	24	2	6	—	38	9	8	—
	pH	4.48	4.95	4.80	4.7	3.88	3.90	3.90	3.40	4.53	4.77	5.6	6.7	4.66	5.14	5.15	5.42
60	Substrate, g.	0.82	1.73	1.73		1.28	0.94	2.08		2.45	1.77	2.79		2.64	1.77	2.69	
	Alcohol, mg.	55	none	84	388	612	123	181	166	133	12	10	none	25	4	23	trace
	Acetic Acid, mg.	none	none	none	none	3	none	6	none	24	none	none	none	none	none	none	none
	Mycelium, mg.	253	11.5	8.0	—	85	173	50	—	7	17	38	—	8	13	11	—
	pH	4.19	4.90	4.66	4.5	3.90	3.95	4.12	3.40	4.20	4.85	5.25	7.30	4.62	4.96	5.25	5.7

Values expressed in g. or mg. per 100 ml. of medium.

In general, as was to be expected, less growth was noted than in aerobic fermentations. (See Table VII.)

This is especially true when we compare the weights of the mycelia synthesized by the organisms. However, more alcohol was accumulated in the absence of oxygen. This is noticeable in the case of Foman, in which, under anaerobic conditions, the alcohol dehydrogenation is slowed down.

When glucose was used as a substrate a yield of more than 80% was usually obtained. The accumulated acetic acid on the 60th day amounts, in the case of Meco, to 6.3% of the glucose disappearing.

This exceeds the value obtained under aerobic condition more than four times.

*Xylose* was also fermented anaerobically giving rise to an alcohol accumulation comparable to the amount obtained in the presence of oxygen. In the case of *Meni* the glucose utilization was slower, resembling the results obtained aerobically.

*Raffinose*, as was pointed out before, generally disappeared faster than glucose in the first 40 days of fermentation with the same organism. However, the glucose utilization was more extensive on the 60th day of analysis. Large quantities of alcohol were accumulated with *Meni* in these fermentations, when carried out under nitrogen.

*Cellulose* in the form of filter paper did give rise to an accumulation of large amounts of alcohol with *Meni* and *Metre* and of a slight amount with *Foman*. The actual extent of cellulose disappearance was not determined. Visual observation indicated, however, an excellent growth with *Meni* and *Metre* and a satisfactory growth with *Foman*. *Meco* did not appear to attack this carbohydrate.

### *Phosphorylation Experiments*

As was observed in this laboratory in the case of trehalose when fermented with *Fusarium lini* B. (21), the rate of dissimilation of raffinose by *Meni* was much higher than the rate of fermentation of glucose. In the case of *Fusaria* it is also known that their saturation with organic phosphorus donors, as compared with yeasts or *B. coli*, is practically insignificant (22). A rather simple chemical splitting instead of the detour of phosphorylation may, therefore, be considered as the major pathway of the enzymatic carbohydrate degradation by these molds *in vivo*.

In some cases, on the other hand, it was experimentally proven (23, 24) that the extent of utilization of a substrate is influenced by the rate of diffusion of that substrate through the cell membrane. In order to attempt a clarification of the observation that a hexose is dissimilated by *Meni* at a slower rate than raffinose, phosphorylation experiments were carried out in the presence of creatine, which could serve as an excellent acceptor for phosphate to be removed via an organic phosphorus donor system present and active in our molds, however unsaturated in this regard they may be.

*Meni* was grown either on glucose or raffinose with three different nitrogen sources—peptone, urea, and asparagine—in the usual, but



enzymatically unfortified medium. At the end of the 17th day of growth the disappearance of glucose, raffinose, pH and mycelial weight were determined (Table VIII). The raffinose disappearance was again more rapid in all three media used than in glucose:

*Experiment No. 1.* On the 17th day the mycelia of Meni grown on glucose and raffinose separately, with asparagine as nitrogen source, were washed, transferred to sterile distilled water containing the same amount of asparagine,  $\text{MgSO}_4$  and 200 mg of creatine hydrate (Eastman Kodak) per liter. After 120 hours the phosphate uptake was measured. No phosphate was found to be accepted by the creatine.

*Experiment No. 2.* On the 23rd day of growth the same experiment was repeated with the exception that 1.5 g./liter of  $\text{KH}_2\text{PO}_4$  was added with the usual 200 mg. of creatine. The phosphate was measured after 12 and 21 hours and again no phosphate uptake was observed.

*Experiment No. 3.* At the 24th day of growth the same amount of creatine was added to the mold left in the original medium and the phosphate measured before and after 12 hours. Again no phosphate uptake was noticeable.

Making due allowance for the unavoidably unsatisfactory conditions of incubation, the presumably rather insignificant amounts of organic P donors present in our molds, and the sensitiveness of the creatine  $\rightleftharpoons$  phosphocreatine equilibrium, it would appear at present that phosphorylation does not constitute an integral part of the phase sequence of carbohydrate degradation by wood-rotting fungi.\*\*

We might, however, venture the assumption that Meni, *without* previous training of the organism, as well as *Fusaria*—in view of the well known fact that cells may differ in their permeability to the same molecular species from 100-fold to 10,000-fold—may permit in the *prevailing dilutions* (a) a more rapid diffusion of some of the higher carbohydrates and that these sugars are (b) fermented directly and, hence, more quickly. This conclusion is amplified by the fact that mycelial weights obtained with Meni are, when grown on peptone-raffinose, even lower than those obtained when grown on peptone-glucose.

\*\* It appears to be noteworthy that Doudoroff (25a), when fermenting trihalose, believes he has demonstrated the presence of an intracellular hydrolytic enzyme in *Ps. Saccharophila* and reasoned that polysaccharides are primarily split by a phosphorolysis. However, in the case of raffinose, the same author (25b) states that practically no hydrolytic or phosphorolytic enzymes catalyzing its breakdown were found in his organism. It also should be noted here that in the case of levulan synthesis by cell free enzyme preparations of *Leuconostoc* (25c)—in which sucrose or raffinose may be the substrate—no mediation of any phosphorylated sugar is required.

TABLE VIII

*Comparison of Glucose and Raffinose Utilization by Meni in the Presence of Different Nitrogen Sources*

Medium	Sugar present		pH		Alcohol	Mycelium wt.
	0 Day	17th Day	0 Day	17th Day	17th Day	17th Day
Glucose, Peptone	3.11	1.91	5.2	4.05	75.4	387
Raffinose, Peptone	3.04	1.53	5.4	3.75	14.6	220
Glucose, Asparagine	3.21	1.92	5.0	3.97	29.6	316
Raffinose, Asparagine	3.19	1.72	5.4	4.1	trace	334
Glucose, Urea	3.02	2.08	6.4	4.7	33.3	364
Raffinose, Urea	3.19	2.05	6.6	4.56	42.0	417

Carbohydrates expressed in g./100 ml. of medium. Alcohol and mycelia in mg./100 ml.

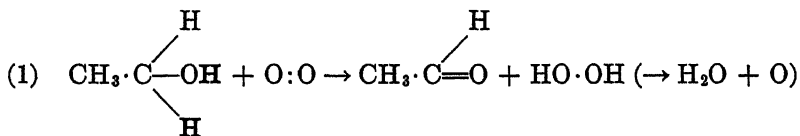
## COMMENT

In the case of plants containing chlorophyll all energy is ultimately utilized to build up organic matter because of absorption of light and its photochemical conversion, while in the case of chlorophyll-free organisms chemical degradations or organic material, *e.g.*, respiration and fermentation, are preponderantly responsible for obtaining the energy required in the formation of new cells. In the case of yeasts and other microorganisms, however, fermentation and respiration as energy-supplying processes are, according to circumstances, coupled with energy-consuming processes, accompanying the synthesis of new cell material. The diversity of ultimate major products present, *viz.*, proteins, fats or pigments enable us to refer to biological *syntheses* of these materials. In the case of alcoholic fermentations, however, the main reaction is the splitting of sugar.

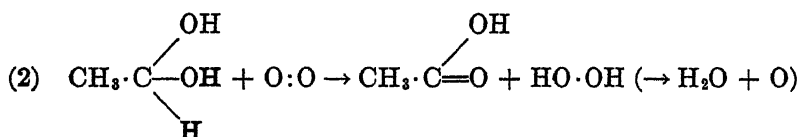
In the present state of our investigation of the carbohydrate metabolism of *Merulii* and *Foman* it would appear that they are perfect alcoholic fermenters. They are able to utilize glucose, xylose, raffinose and cellulose, producing stoichiometric amounts of alcohol from the first three carbohydrates aerobically. However, similarly to the *Fusaria* fermentation of trehalose, the dissimilation of raffinose with *Meni* proceeds more rapidly than that of glucose, whereby phosphorylation does not seem to precede the alcohol formation. It was also noticed in all cases that the ethyl alcohol so formed is subject to a dehydro-

genation, whereby substantial amounts of acetaldehyde and acetic acid could be isolated and identified. The formation of succinic acid was also ascertained. Presumably due to a lack of close integration of the enzymatic activity in these molds, it is not surprising that investigators often failed to ascertain intermediary and terminal products in this type of degradation. Only frequent and painstaking analyses enable the student to isolate the compounds.

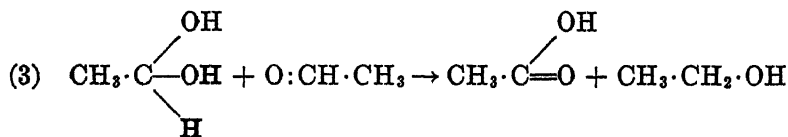
The isolation of acetaldehyde without interception, in distinction to experience with other (26) molds, and of acetic acid, the accumulation of the latter under anaerobic conditions amounting to four times that obtained aerobically, is significant and raises the question of the origin of these products. Although the disappearance of ethanol, when given as a carbon source, points to its direct dehydrogenation, namely:



it would still be conceivable that two processes are progressing simultaneously, *viz.*, (a) a dehydrogenation of the acetaldehyde so formed with the participation of oxygen:



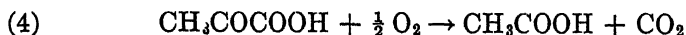
or, and more preponderantly so under anaerobic conditions, (b) a dismutation of two molecules of acetaldehyde:



resulting in the formation of acetic acid and alcohol.

However, an uncertainty enters into the picture. As was pointed out in the experimental part, addition of thiamine to the medium was found necessary to obtain satisfactory growth on our semisynthetic media. Our inability to isolate or identify pyruvic acid as an inter-

mediary of the carbohydrate breakdown, due to the failure of these fungi to reduce nitrates, in contrast to the mechanism of the carbohydrate breakdown by *Fusaria*, also points to the possibility of an oxidative decarboxylation of pyruvic acid, *viz.*:



which may have occurred, even if only as a side reaction. Be it at present as it may, the fact that the formation of formic acid was never observed, suggests, however, the improbability that a hydroclastic split (27) could have taken place as a bypath.

As to the phase sequence of the anaerobic degradation of cellulose by Meni, Metre and Foman the evidence so far available shows that, in addition to ethanol, traces of acetic acid were found. This is at variance with the mechanism of a cellulose fermentation by anaerobic or thermophilic bacteria as outlined by Lyman and Langwell (28) or Neuberg and Cohn (29), who indicate that the degradation proceeds similarly to the phases of a butyric acid-acetic acid fermentation.

It would appear that, in our cases, we are confronted with a course of carbohydrate fermentations analogous to those occurring during the degradation of the composite sugars mentioned above, with the distinction that the accumulation of transitory and terminal products seems to be inhibited by the limited diffusion of the carbohydrate to be acted upon by the enzymes present.

The overall picture of this preliminary study would again clearly indicate the striking differences which exist in the major pathways of carbohydrate breakdown and dehydrogenations between yeast on one hand (30) and a number of molds on the other. The results, moreover, further underline the necessity of recognizing the diversified actions and varied phase sequences which occur during the course of degradations with different intact cell systems. Consequently, pompous statements in regard to "general pathways" of biochemical reactions appear to be thoughtless when examined within the narrow field of vision (31) delineated by some chemo-autotrophic bacteria.

#### ACKNOWLEDGMENT

It is a pleasure to acknowledge the assistance received from the Rockefeller Foundation and Burton T. Bush, Inc., during the course of this study. All wood samples were obtained through the courtesy of Givaudan-Delawanna, Inc., Delawanna, N. J. We are under obligation to Corn Products Refining Co., New York, for a generous sample of inositol.

## SUMMARY

1. The carbohydrate metabolism of the four wood-rotting fungi, *Merulius niveus*, *Merulius tremellosus*, *Merulius confluent* and *Fomes annosus*, was studied.
2. The fungi supported a satisfactory growth when thiamine was added to the media.
3. The organisms grew in a pH range of 3 to 8, the optimum pH being between 4.5 and 7.
4. Peptone, asparagine and urea were the best organic nitrogen sources for growth.
5. Inorganic nitrogen proved capable of supporting only a scanty growth.
6. Glucose, xylose, raffinose and cellulose, as well as different kinds of wood, can be utilized by these organisms.
7. Among the metabolic products found to be accumulating were: ethyl alcohol, acetaldehyde, acetic acid and traces of succinic acid.
8. From the metabolic products of Foman a compound was isolated which gave a 2,4-dinitrophenylhydrazone melting at 208°C.
9. The course of the alcoholic fermentation of glucose, xylose, raffinose and cellulose was studied aerobically as well as anaerobically.
10. Ethanol, when given as a substrate, is quickly dehydrogenated at varying rates by all four fungi.
11. Cellulose, when fermented under aerobic condition with these fungi, gave rise only to traces of alcohol.
12. In cellulose fermentation under nitrogen ethyl alcohol was found to accumulate with Meni, Metre and Foman.
13. Raffinose was utilized more rapidly than glucose with Meni.
14. Phosphorylation experiments, utilizing creatine as a phosphorus acceptor, were carried out with Meni in the presence of glucose or raffinose as carbohydrate source.

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## On the Mechanism of Enzyme Action.

### Part 28. Application of Resazurin to the Study of Dehydrogenations by Certain *Merulii* and *Fomes Annosus*<sup>1</sup>

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#### INTRODUCTION

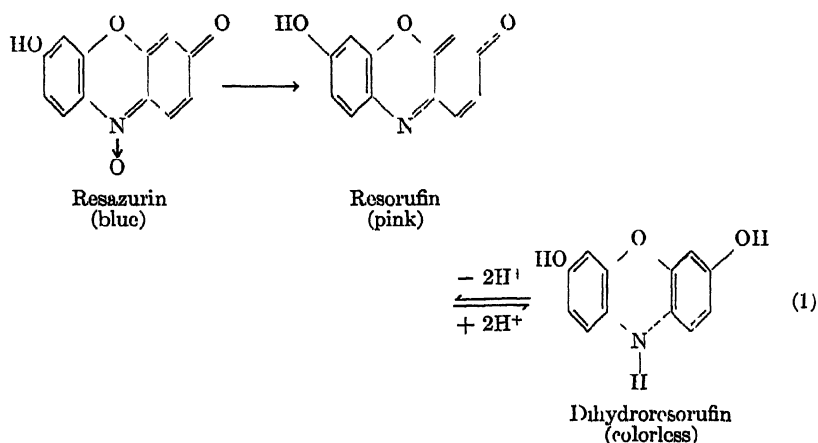
The embodiment of an efficient dehydrogenating enzyme system by the four wood-destroying molds: *Merulius niveus* (Meni), *Merulius tremellosus* (Metre), *Merulius confluens* (Meco) and *Fomes annosus* (Foman) has been demonstrated by previous investigations in this laboratory (1). Similar observations were also made in studies with *Fusaria* in which especially oxygen, different nitrates, or even elementary sulfur were able to serve as hydrogen acceptors (2). The reduction of these acceptors, in distinction to the mechanism of the hydrogen transfer itself, appeared to be a single stage reaction in all cases thus far studied. In the case of the conversion *D*-Sorbitol → Sorbose → 2-keto-*L*-gulonic acid we would be dealing with a more complex system.

It, therefore, appeared desirable to utilize a dye which could act as an interagent in visualizing the progress in a two stage reaction, and would simultaneously serve as an indicator in a straight dehydrogenation—*e.g.*, isopropyl alcohol → acetone, presumably feasible on applying the enzyme system of our wood-rotting fungi. The dye selected for this purpose was *resazurin* (3), which was used previously (4) for estimating the bacterial content of milk. Resazurin passes through the following stable stages during reduction:

<sup>1</sup> For a preliminary report see: *Nature* 157, 355 (1946). Presented before the Division of Sugar Chemistry and Technology of the Am. Chem. Soc., Atlantic City, N. J., April, 1946.

<sup>2</sup> Communication No. 45.



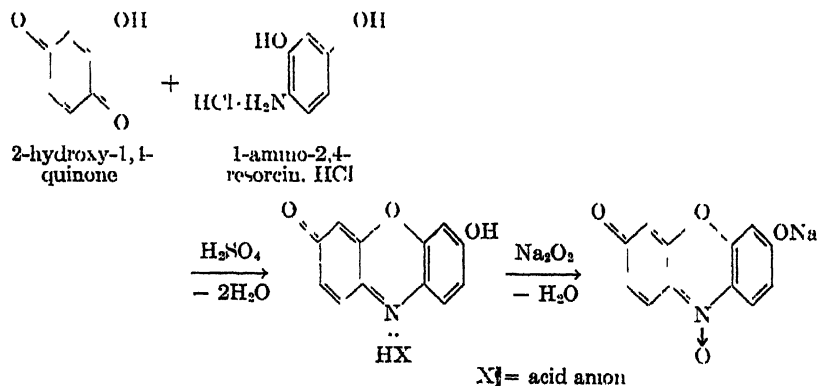


This represents, in the second step, a reversible oxidation-reduction system, without apparently giving rise to the formation of semi-quinones (5).

#### SYNTHESIS OF RESAZURIN

Inasmuch as the procedure of Nietzki (6) for the preparation of resazurin provides for the possible admixture of some resorufin, the presence of which at the beginning of the dehydrogenation reaction could anticipate its enzymatic formation, it was deemed necessary to attempt a synthesis which would enable us to offset the probability of simultaneous formation of this contaminant.

The synthesis is based on the following reaction:



The utilization of a strong oxidizing agent, sodium peroxide, excludes the possibility of the formation of resorufin and supplied us with the desired uniform product.

*Preparation of Resazurin.* To a suspension of 12.4 g. (0.1*M*) of 2-hydroxy-1-4-quinone (7) and 16.2 g. (0.1*M*) of 1-amino-2,4-resorcin hydrochloride (8) in dry ether, contained in a round bottom flask fitted with a mercury-sealed stirrer, reflux condenser and dropping funnel, 100 g. of conc.  $\text{H}_2\text{SO}_4$  are slowly added dropwise with continuous stirring. When the reaction has subsided, a slight excess of acid is added and the refluxing and stirring continued for an additional half hour. After cooling in an ice bath, solid sodium peroxide is carefully added until the reaction mixture is basic to phenolphthalein paper. The ether is then removed *in vacuo* and the residue taken up with water and filtered. The filtrate is evaporated to dryness *in vacuo*. The crude material is then taken up with absolute ethanol. After filtering off any impurities, this solution is evaporated to dryness *in vacuo*, yielding the sodium salt of resazurin. Yield of raw product 20% of theoretical. The compound is purified by solution in hot water, followed by addition of hot sodium carbonate solution, precipitating the sodium salt. This procedure is repeated three times to obtain a very pure product. Analysis: N calculated 5.57%, N found 5.27%.

This preparation satisfied the requirements of the dehydrogenation studies.

#### *Dehydrogenation of d-Sorbitol and Isopropyl Alcohol*

The spore-mycelial suspensions used in these experiments were the same as in the previous (1) study and were derived from the same cultures. Preliminary experiments were run by growing the molds in 125 ml. Erlenmeyer flasks. Each flask contained 50 ml. of nutrient medium, was sterilized at 15 lbs. pressure for 20 minutes and allowed to grow in the dark at 28°C. In utilizing d-sorbitol as a substrate, a 2% concentration of the crystalline alcohol was employed and was added to the medium prior to sterilization. In the case of isopropyl alcohol, a 0.4% concentration was used and mixed with the sterilized salt solution with sterile technique. The concentration of the dye was  $1.5 \times 10^{-5}$  *M* and was placed in the medium prior to sterilization.

In utilizing fully developed mats the technique was as follows: the mold was grown on the d-glucose nutrient medium mentioned previously (1), and 50 ml. of this solution were placed in a 125 ml. Erlenmeyer flask. After a two week growth the medium was poured off and the mat then washed several times with 50 ml. portions of sterile distilled water. After each washing the water was decanted, maintaining sterile conditions at all times, and the mat then transferred to a 400 ml. Fernbach flask containing 250 ml. of the substrate medium desired. Mycelial weights obtained in spore-mycelial suspension experiments were determined according to the usual procedure, as heretofore.

*Analytical Methods.* The qualitative course of the dissimilation of d-sorbitol by the four molds was established by the following facts: (1) a positive Seliwanoff test was obtained, indicating ketose forma-

tion; (2) discoloration of resazurin indicated the progress of the reaction according to equation (1), and (3) Munson-Walker values for reducing sugar. This last method was also employed for quantitative determination of the dissimilation product.

The course of conversion of isopropyl alcohol was ascertained by means of the vanillin (9) and *o*-nitrobenzaldehyde (10) reactions with acetone. The latter was, moreover, identified by its 2,4-dinitrophenylhydrazone, m.p. 125–126°C. Acetone was quantitatively determined in the presence of isopropyl alcohol by the iodoform reaction (11).

### *Redox Potentials in Meni-Systems in the Presence of Resazurin*

For redox potential measurements the fully grown mat of *Meni*, possessing the strongest dehydrogenating system of the molds studied, was utilized as a source of enzyme material. To offset the effect of oxygen on the dye it was obligatory to carry out the measurements anaerobically.

To effect this the following technique was employed: The mat was transferred to a 125 ml. Erlenmeyer flask containing 100 ml. of sterile medium plus resazurin. The cotton plugs were then replaced with previously sterilized rubber stoppers fitted with inlet and outlet tubes, the inlet tube being extended to the surface of the medium, and the two tubes connected by means of a short piece of rubber tubing. Nitrogen gas from a tank,<sup>1</sup> after passage through a mercuric chloride solution, was used to displace the air in the flasks.

Redox potential measurements were made against a saturated calomel electrode and the values are expressed against a normal hydrogen electrode. While making the measurements the space above the mats was flushed with nitrogen gas to maintain anaerobic conditions.

At the outset of these measurements it was thought conceivable that the yield of dehydrogenation products to decolorization of the dye and redox potential might be related. It should, however, be borne in mind that a system consisting of a *growing* organism—in contrast to that used, for instance, by Michaelis and Smythe (13)—of a dehydrogenating enzyme system therein present, and of an indicator which could not be applied in any stoichiometric amounts, makes it difficult to refer the recorded data to the requirements of a normal redox potential curve of resazurin. It may be true, however, that the

<sup>1</sup> The absence of *measurable* amounts of oxygen could only be guaranteed by using nitrogen gas purified by the technique of Meyer (12). This degree of anaerobicity, though highly desirable, was, of course, not attained.

initial stage of the enzymatic reaction was accelerated by the small amounts of resazurin present.

Although the decolorization of resazurin is complete in a few days, in both cases, and, accordingly the  $\left[ \frac{\text{oxid.}}{\text{red.}} \right]$  ratios are comparable, the shapes of the curves (Fig. 1) obtained in the presence of *d*-sorbitol

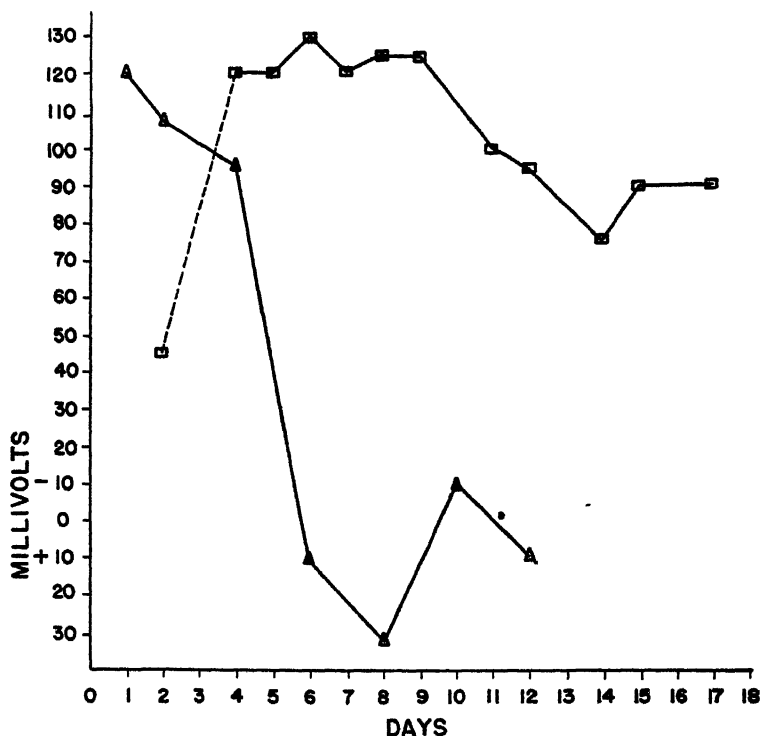


FIG. 1

Redox Potential Measurements

- 0.4% Isopropyl alcohol +  $1.5 \times 10^{-5} M$  resazurin with Meni mats  
 △ 2.0% *d*-Sorbitol +  $1.5 \times 10^{-5} M$  resazurin with Meni mats

and isopropyl alcohol deviate significantly from each other. This difference may be attributed to the intrinsic dynamics of the total system as stated above, due to the growth of the organism itself and discrepancies in the growth conditions.

## RESULTS AND DISCUSSION

*Dehydrogenation of d-Sorbitol.* The results obtained with this substrate corroborate our findings on the presence of a dehydrogenating enzyme system in the four wood-destroying molds. The dehydrogenation of *d*-sorbitol was ascertained in one aspect, by means of decolorization of resazurin. The rapid rate of decolorization of the dye, when incorporated in the medium inoculated with *Meni*, together with the

TABLE I  
*Aerobic Dehydrogenation of d-Sorbitol*<sup>1</sup>

Day	<i>Meni</i>		<i>Metre</i>		<i>Meco</i>		<i>Poni m</i>	
	Mycehal wt	Sorbose	Mycehal wt	Sorbose	Mycehal wt	Sorbose	Mycehal wt	Sorbose
	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml	mg /100 ml
7	23.6	None	17.4	None	13.6	None	28.0	None
12	150.0	None	30.4	None	25.8	None	209.2	None
15	236.4	None	55.6	None	78.6	None	226.8	None
19	276.0	24	80.0	None	61.0	None	249.2	8
21	299.0	30	88.6	None	72.0	Trace	283.0	8
24	314.0	36	87.2	None	114.4	Trace	288.2	8
26	356.0	40	124.8	None	152.6	8	292.8	8
28	259.2	40	159.4	None	211.2	8	309.6	8
30	225.4	40	164.4	None	222.4	8	253.0	22
32	175.2	40	184.6	Trace	282.0	8	240.8	14
36	143.0	40	238.2	Trace	234.0	14	214.0	22
40	132.0	40	225.2	4	278.8	14	210.4	22
52	108.8	42	303.6	4	420.0	22	199.2	22
56	102.2	56	324.6	None	350.8	16	-	22
60	91.0	56	300.0	None	395.4	16	195.4	22

<sup>1</sup> 2% crystalline *d*-sorbitol medium inoculated with spore-mycelial suspension.

larger amounts of reducing substances accumulated, attest to the ease with which the first step of this important conversion (see p. 440) can be carried out. A quantitative comparison of the action of the four molds on *d*-sorbitol is presented in Table I. As preliminary experiments established the decolorization of resazurin, it was thought interesting to investigate the effect of the dye on the growth of the organisms. The findings of such a study are listed in Table II. Comparing the results in Tables I and II it is evident that the dye has an inhibitory

effect in the case of Metre and Meco. Mycelial weights are lower and no appreciable amounts of reducing substances are detected. The Meni-inoculated medium does not seem to be greatly affected by the dye although its growth does appear to be slightly retarded at the outset.

Regarding the visible characteristics of the sorbitol medium supplemented with resazurin, it was observed that complete decolorization of the dye was effected when a heavy mycelial mat had formed, thus eliminating the effect of atmospheric oxygen which tends to cause

TABLE II

*Aerobic Dehydrogenation of d-Sorbitol in the Presence of Resazurin*<sup>1</sup>

Day	Meni		Metre		Meco	
	Mycelial wt.	Sorbose	Mycelial wt.	Sorbose	Mycelial wt.	Sorbose
	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.
15	158.0	14	21.4	None	16.0	None
18	205.2	14	27.2	None	21.0	None
20	263.0	14	43.4	None	28.2	None
22	266.8	16	—	None	—	None
25	350.0	29	36.8	Trace	33.2	Trace
29	273.4	40	52.6	Trace	36.0	Trace
39	208.6	40	75.6	None	46.8	None
41	190.0	40	88.0	None	54.0	None
45	113.4	40	98.0	None	60.4	None
50	104.8	48	124.0	None	130.4	None

<sup>1</sup> Medium same as in Table I +  $1.5 \times 10^{-6}$  M resazurin. No measurements were made with Foman because of complete inhibition of growth by the dye.

reversion of the dye to its original blue color. Any dye trapped on the surface of the mycelial mat retains its original blue color for the reason just cited, but the dye contained in the solution below the mat surface becomes decolorized.

Further inspection of Tables I and II, however, discloses the additional fact that while there is a decrease in mycelial weight, the reducing compound content continues to increase. This would lead one to assume that, due to the apparent "starvation" of the mycelium, the ability of the dehydrogenases present to act upon *l*-sorbose in the manner previously mentioned is destroyed, simultaneously preventing formation of the 2-keto-*l*-gulonic acid. That *l*-sorbose itself can be

TABLE III  
*Aerobic Dissimilation of Sorbose<sup>+</sup>*

Day	Meni		Metre		Mero		Roman	
	Mycelial wt	Sorbose Disappearing	Mycelial wt	Sorbose Disappearing	Mycelial wt	Sorbose Disappearing	Mycelial wt	Sorbose Disappearing
	<i>mg/100 ml.</i>		<i>mg/100 ml</i>		<i>mg/100 ml</i>		<i>mg/100 ml</i>	
22	296.0	1400	30.6	150	5.2	50	23.8	300
29	291.4	1060	19.4	200	7.0	50	83.0	300
38	363.2	1780	24.6	250	15.0	200	97.2	550
42	243.6	2300	—	—	—	—	—	—

\* Medium inoculated with spore mycelial suspension.

\* Initial sorbose concentrations 2400 mg./100 ml.

dissimilated by the four molds is demonstrated by experimental data presented in Table III. Although growth was slow in the case of Meni practically all the sorbose disappeared without giving rise to a definite product.

The pertinent fact of very slow growth of the microorganisms on a sorbitol medium prompted the study of its dissimilation in the presence

TABLE IV  
*Comparison of Yields of Sorbose Obtained with a Spore-Mycelial Suspension or Fully Grown Mats of Meni*

Day	Suspension		Mats
	Mycelial wt. <i>mg./100 ml.</i>	Sorbose <i>mg./100 ml.</i>	Sorbose <i>mg./100 ml.</i>
21	257.2	19.5	28.0
28	285.4	32.0	29.0
30	328.2	27.0	35.0
33	160.4	31.0	33.0
35	144.6	27.5	28.5

of an abundant supply of enzyme in the form of a fully grown mycelial mat. The technique employed is described in the experimental part and the results are compared with those obtained from spore mycelial suspension studies in Table IV. In this phase of the work only Meni was employed as it is the most luxuriantly growing mold of the group.

The results of this experiment again illustrate the relative enzymatic unsaturation of the mold as only after an initial incubation period of

15-18 days is the reducing substance qualitatively detectable. And, too, there is continued growth of mycelium inasmuch as a heavy mat is formed.

*Dehydrogenation of Isopropyl Alcohol.* As in the case of sorbitol, the path of the dissimilation of isopropyl alcohol by the four molds was established by decolorization of resazurin when added to a 0.4% medium of the alcohol. Here too, as growth was very slow it was deemed suitable to utilize the fully grown Meni mat to supply an

TABLE V  
*Aerobic Dehydrogenation of Isopropyl Alcohol \**

Day	Meni	Metre	Meco	Foman
		<i>Acetone, mg./100 ml.</i>		
10	5.9	6.0	traces	10.9
14	17.4	8.9	traces	14.4
18	25.3	17.3	6.9	27.8
20	34.9	11.2	9.4	21.3

\* 0.4% Isopropyl alcohol medium.

abundant source of the dehydrogenating enzymes. In Table V the yields of acetone are recorded. In this case the medium was inoculated with the usual spore-mycelial suspension. The results obtained with isopropyl alcohol, using fully grown mats, are much higher than those from the suspension (Table VI), in contrast to results obtained with sorbitol.

An additional observation in the study of the dissimilation of isopropyl alcohol by these molds is that, while the dye is changed to pink, it is not completely decolorized, for, as long as a heavy mycelial

TABLE VI  
*Aerobic Dehydrogenation of Isopropyl Alcohol with Meni Mats \**

Day	Acetone <i>mg./100 ml.</i>
3	54.5
8	74.3
10	119.0
12	141.2
14	151.0
19	118.0
25	72.4

\* 0.4% Isopropyl alcohol medium.



mat is not formed, there cannot be satisfactory exclusion of atmospheric oxygen, the presence of which allows for the reversion in color and chemical change between resorufin and dihydroresorufin.

Inspection of the values recorded in Table VII shows that isopropyl alcohol can also be dissimilated anaerobically, as compared with sorbitol which cannot.

It should also be mentioned that, in contrast to earlier findings (14) with *Fusaria*, the molds employed in the present study did not give rise to the hydroclastic formation of formaldehyde as a secondary dissimilation product of isopropyl alcohol.

TABLE VII  
*Anaerobic Dehydrogenation of Isopropyl Alcohol with Meni Mats*<sup>1</sup>

Day	Acetone (mg./100 ml.)
1	11.0
2	14.5
3	6.0
4	None
5	30.9
6	40.0
7	None
8	None
9	21.8
10	21.8
11	13.9
12	None
13	14.5
14	None

\*  $1.5 \times 10^{-5}$  M resazurin added to medium.

Furthermore, it should be noted that the influence of the steric configuration, according to Bertrand's rule (15), alters the course of the dehydrogenation. Thus, while it is obvious that acetone would result from the dehydrogenation of isopropyl alcohol, the formation of *l*-sorbitol from *d*-sorbitol depends on a *cis*-configuration of the alcoholic hydroxyl groups on carbons 2 and 3, so that, instead of the primary alcohol groups at each end of the sugar molecule being dehydrogenated, only the secondary alcohol grouping is attacked and converted to the ketone, while the observed formation of 2-keto-*l*-gulonic acid from *d*-gluconic acid (16) seems to be a deviation, our findings are in agreement with the rule.

## ACKNOWLEDGMENTS

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## SUMMARY

1. The dehydrogenation of *d*-sorbitol and of isopropyl alcohol by the four wood-destroying molds *Merulius niveus*, *Merulius tremellosus*, *Merulius confluens* and *Fomes annosus* was studied.

2. From *d*-sorbitol small amounts of *l*-sorbose, and from isopropyl alcohol larger amounts of acetone, were obtained.

3. Resazurin proved a convenient indicator in dehydrogenation studies.

4. A new synthesis of resazurin was presented.

5. Redox potentials were measured of the two systems Meni-*d*-sorbital-resazurin and Meni-isopropyl alcohol-resazurin.

6. Sorbose obtained from the dissimilation of *d*-sorbitol could not be further converted by the "starving" organism in contrast to its utilization by the growing organism.

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## Compounds Active in Correcting Sulfonamide-Induced Granulocytopenia in Rats

Sirs:

The most recently synthesized vitamin of the B-complex, variously designated as the "*L. casei* factor," "vitamin B<sub>6</sub>," "folic acid," etc., is known to exist in a number of closely related forms. Four compounds possessing qualitatively the same activity for the rat (and for the chick) have now been isolated; one from liver (1) (2), two from yeast (2) (3) and one from a fermentation residue (4). One of the four has been synthesized (5). A closely related substance which occurs in spinach (6) (7) may differ from each of these four (4) and may constitute, therefore, a fifth form of the vitamin. The compound in yeast which was recently isolated in crystalline form (3) is probably identical with the one which we described earlier (8) (9). Data in support of this point of view will be given at this time. In addition, we wish to present evidence for the existence of a compound in liver, previously undescribed, which possesses activity for rats similar to that of each of the known forms of the *L. casei* factor.

Data given in the table show that the concentrate from yeast (10) previously described (8) (9) possesses less than 0.4% as much microbiological activity as might be expected from its therapeutic effect in rats with sulfonamide-induced leucopenia and granulocytopenia (11). As indicated, the microbiological activity is greatly enhanced by treatment with acid, alkali or an enzyme preparation from rat liver. These properties agree only partially with those of "vitamin B<sub>6</sub> conjugate" as originally described (12) which must, however, have been a mixture (13). The properties of our concentrate agree very well with those of crystalline "vitamin B<sub>6</sub> conjugate" (3), both as to the ratio of microbiological potency to activity in experimental animals and, qualitatively, as to the enhancement of microbiological potency by simple procedures.

	$\gamma$ <i>L. casei</i> factor (by <i>S. faecalis</i> assay), per "rat unit"
Synthetic <i>L. casei</i> factor (5) (18)	8.
Yeast concentrate (10)	0.03 <sup>+</sup>
Liver concentrate (14)	0.3

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	<i>S. faecalis</i> assay values after treatment (expressed as per cent of "before treatment" values)
Yeast concentrate (10)	
Autoclaved 2 hrs. at 15 lbs. pressure, pH = 3 <sup>++</sup>	800
Autoclaved 2 hrs. at 15 lbs. pressure, pH = 14 <sup>++</sup>	750
Incubated with enzyme preparation from rat liver (15) <sup>++</sup>	1000
Liver concentrate (14)	
Autoclaved 2 hrs. at 15 lbs. pressure, pH = 3	60
Autoclaved 2 hrs. at 15 lbs. pressure, pH = 14	120
Incubated with enzyme preparation from rat liver (15)	70
Incubated with enzyme preparation from almonds (17)	120
Incubated with enzyme preparation from almonds (19)	120
Incubated with enzyme preparation from chicken pancreas (20)	90
Incubated with fresh whole rat liver	110
Incubated with takadiastase, 24 hrs.	80
Incubated with pepsin 24 hrs., then trypsin 24 hrs.	70
Incubated with pepsin 96 hrs., then trypsin 24 hrs.	40

\* The term "rat unit" is used here to indicate the minimum amount which must be given daily for 4 days to a rat with sulfonamide-induced granulocytopenia to cause a full granulocyte response (from 4000 or fewer total leucocytes and 200 or fewer polymorphonuclear granulocytes to 10,000 or more total leucocytes and 2000 or more granulocytes per cu. mm.).

\*\* The ratio of activities of this concentrate (10) for *S. faecalis* and for the rat varied from batch to batch. The lowest "rat unit" value repeatedly obtained was 0.03  $\gamma$ . The particular batch of concentrate used for treatment with acid, alkali and enzyme had the unusually high original "rat unit" value of 0.3  $\gamma$  and the indicated increases are from this base.

Further data in the table indicate that there is present in certain liver concentrates (14) a compound which has not previously been described. While possessing activity for granulocytopenic rats, these concentrates neither exhibit the expected microbiological potency *nor can this potency be materially increased by treatment with acid or alkali or with the usual enzyme preparations (15) (16) or with proteolytic enzymes*. To date we have found no method which is effective in "activating" (for microorganisms) the compound (or compounds) present in these liver concentrates.

Either we have the curious situation that the granulocytopenia may be corrected by either one of two different vitamins or else we are dealing with an hitherto undescribed form of *L. casei* factor, which cannot be made available to microorganisms by any procedure which we have tried. If the latter alternative is correct, then present microbiological assay methods for *L. casei* factor, even those which include treatment of the material being assayed with "vitamin B<sub>6</sub> conjugate" (17) may, for some tissues, yield highly erroneous results.

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### Note on the Metabolism of Some Ribose Derivatives

It was observed that incubation of purine nucleotides and nucleosides with tissue extracts changes the ribose group in such a way that it can no longer be determined by the quantitative orcinol reaction (1). This is due to the combined action of several enzymes which are found in the following tissues in decreasing concentration: liver, kidney, spleen, brain, blood, heart, and striated muscle. Free ribose and the other pentoses are not changed under our experimental conditions. Adenosine and guanosine are broken down somewhat more rapidly than adenosine-5-phosphate, adenosine-3-phosphate, cozymase, and inosinic acid. Since the above listed tissues are known to be rich in phosphatases, it seems likely that the first step is dephosphorylation of nucleotides to nucleosides. The nucleosides are acted upon by nucleosidase, yielding ribose-1-phosphate (2). We can confirm Kalkar's finding that phosphate is taken up in this process, but we find that the labile ribose-1-phosphate is only an intermediary product, which is stabilized rapidly. The pentose concentration of a sample containing 11.6 gamma moles guanosine per ml. fell to 7.5 gamma moles after 1 hour, and to 2.4 gamma moles after 3 hours incubation at 37°. After 1 and 3 hours, 3.5 gamma moles and 9.7 gamma moles of inorganic phosphate had been converted to the ester. The compound formed is stable toward acid, in contrast to ribose-1-phosphate. A comparison with other ribose phosphoric acid esters showed that the pentose reaction vanishes rapidly when ribose-5-phosphate is used as a substrate, while ribose-3-phosphate reacts very slowly or not at all. From incubation mixtures containing 10.8 gamma moles of ester, 4.4 gamma moles of ribose-5-phosphate disappeared in one hour, while ribose-3-phosphate remained unchanged. It occurred to us that an enzymatic rearrangement of ribose-1-phosphate to ribose-5-phosphate (analogous to phosphoglucomutase action) might be the most reasonable explanation of our finding. In absence of phosphate no pentose disappears from the incubation mixture, when nucleosides are used as



substrate. In the breakdown of ribose-5-phosphate, the concentration of free phosphate in the medium remains constant. The disappearance of the ribose ester does not depend on the presence of oxygen and is not inhibited by fluoride and iodoacetate. It is probable, however, that our dialyzed enzyme extract is an incomplete system and that an intermediate compound is accumulated.

We are attempting to identify the degradation products beyond ribose-5-phosphate. In addition to the prospects of elucidating the fate of ribose in higher organisms, our observation has significance for the methods of nucleotide determination which involve the orcinol test.

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## Book Reviews

**Advances in Carbohydrate Chemistry.** Volume I, Edited by: W. W. PIGMAN and M. L. WOLFROM. Academic Press Inc., Publishers, New York, N. Y. 1945. xii+374 pp. Price \$6.00.

On the foundations so truly laid by Emil Fischer there has been erected in the course of recent years a vast edifice of carbohydrate chemistry, the implications of which are becoming of ever increasing importance in medicine and biology and in industry. It is becoming more and more difficult for the specialist and non-specialist alike to keep track of all the literature, and in this volume, which is a new venture and the first of a projected series of similar collections, there is to be found a group of articles in which our knowledge of certain specialized branches of carbohydrate chemistry is summarized and discussed.

The book begins, very appropriately, with an article by C. S. Hudson on "The Fischer Cyanohydrin Synthesis and Configurations of Higher Carbon Sugars and Alcohols." In this fascinating chapter is given an historical as well as a chemical account of the sugars and sugar alcohols including some which contain nine carbon atoms. Using rules based on extensive experience, Dr. Hudson is now able to suggest in advance the most satisfactory methods for the synthesis of these substances and details a simple nomenclature to characterize the configuration of these sugars. This article is followed by an account of "The Altrose Group of Substances" by Nelson K. Richtmyer. At one time the altrose group of substances, which are derived from the rare pentose, ribose, were comparatively unknown. The author describes their preparation from such common substances as lactose and cellobiose, and discusses the rare septose, sedoheptulose in which the altrose configuration occurs. The chapter concludes with methods of preparing derivatives of altrose from pectin. A useful list of derivatives of d- and l-altrose is included in the article.

The carbohydrate orthoesters are dealt with by E. Pacsu and the thio and seleno sugars are discussed by Albert L. Raymond. So little is known about these interesting and unusual thio-sugar derivatives that they may well repay further study both from a botanical and from a chemical viewpoint.

"The Carbohydrate Components of the Cardiac Glycosides" have recently been the subject of several publications and in this very interesting article, by Robert C. Elderfield, the occurrence, preparation and constitution of all the known simple sugars, and of one of the disaccharides, is discussed. It is certain that much further work will be required before the final elucidation of the complex structure of these rare and interesting sugars is decided.

C. Jeleff Carr and John C. Krantz discuss in some detail the "Metabolism of the Sugar Alcohols and their Derivatives," and an exceedingly interesting and readable account of "The Chemistry of the Nucleic Acids" has been contributed by R. Stuart Tipson, who was associated with the late Doctor P. A. Levene, a pioneer in the

field of nucleic acids and the first to isolate from them *d*-ribose. An account is given of the chemistry of *d*-ribose and the proof that this sugar exists in the furanose form in the nucleic acids. The difficulties encountered in the examination of the desoxy-ribosylpurines are discussed, and the isolation of *d*-2-desoxyribose which crowned Levene's work is described. The chapter concludes with a short account of the physical chemistry and polymeric properties of the nucleic acids.

Perhaps in no other branch of carbohydrate chemistry have such advances been made in recent years as in the chemistry of starch. In one of the articles now under review T. J. Schoch, who is himself one of the main contributors on new knowledge of this subject, gives an account of the "Fractionation of Starch" and its separation into amylose and amylopectin, an operation which is likely to become of increasing industrial importance in the near future owing to the very different and desirable physical properties of the two components.

The next two articles are by Roy L. Whistler on "The Preparation and Properties of Starch Esters," and by Charles R. Fordyce on "Cellulose Organic Esters." These two articles deal mainly with the methods of preparation and physical properties of the respective polysaccharide esters.

The volume ends with an article on "A Discussion of Methods of Value in Research on Plant Polyuronides" by Ernest Anderson and Lila Sands. The plant polyuronides are probably some of the most complex polysaccharides known. In this very useful article are collected together practical methods for the isolation and purification of the hemicelluloses, mucilages, gums, etc. Details are also given for their hydrolysis and the identification of the resulting sugars. Many references are given to original papers.

The volume should serve a most useful purpose, and the Editors are to be congratulated on having succeeded in their object of obtaining articles which are at one and the same time critical and integrative in outline and intelligible to the non-specialist. In one or two instances new and hitherto unpublished observations are recorded in this volume, but it is to be hoped that the Editorial Board will see to it that "Advances in Carbohydrate Chemistry" will be maintained as a review and not as a medium for the publication of original research, the latter function being, in the reviewer's opinion, inappropriate to this type of work.

E. L. HIRST, Manchester, England

**Physical Chemistry of Cells and Tissues.** By RUDOLPH HÖBER, with the collaboration of DAVID I. HITCHCOCK, J. B. BATEMAN, DAVID R. GODDARD, and WALLACE O. FENN. The Blakiston Company, Philadelphia and Toronto. 676 pages. \$9.

The pathway for progress in the biological sciences is two-fold. One way, necessarily of earlier origin and pre-requisite to the search for another way of approach, is the accumulation of facts obtained by observation and experiment, disregarding their explanation in terms of the fundamental laws of physics and chemistry, such as morphology, macro- and microscopic, chemical identification of the constituents of cells and tissues, descriptive physiology, physical and chemical, etc. Once a certain level of knowledge has been accumulated, another approach opens spontaneously: *i.e.*, to integrate all the data obtained with acknowledged fundamental laws of physics and chemistry. The first approach is termed by the editor of this book, "physiology

from above," the other, "physiology from below." The attempt to enter the second route implies the assumption that the living organism has at its disposal no other forces than those valid in inorganic matter, and that all the complexity of the physico-chemical achievements of the cell can be attributed to the complexity of the physico-chemical system which it represents and not to specifically vital forces or agents alien to non-living matter. This book is an attempt to set forth the present level of progress of physiology from below.

More than forty years ago, the editor, who has himself contributed several chapters, published his "Physikalische Chemie der Zelle und Gewebe," which may be considered as the first systematic treatise of this kind of physiology. It was a landmark in biological research, written with the enthusiasm of youth and encouraged by the then novel applications especially of the laws of osmosis and electromotive force to cellular physiology. The present book is the long-desired attempt to bring such a presentation up to date. The difference in the situation is that there is now no single man who might be in command of all branches of science pertaining to the task. The distribution of the material among various authors will of necessity render the presentation somewhat inhomogeneous. This cannot be helped.

The book may be thought of as consisting of two moieties. The first, shorter one, is a treatise of those parts of physics and physico-chemistry which are expected to play an essential role in the second part dealing with the actual biological facts ascertained by observation and experiment. Thus, the ideal state of affairs would prevail if the second part would explain the biological parts by reference to laws set forth in the first part. He who expects this proposition to be materialized will be disappointed. However, this is not the fault of the book but of the low level of knowledge attained so far. The references in the second half of the book to specific statements of the first half, are rather scarce indeed. They are encountered more in the remarks introductory to each of the later sections than in their bulk. It is hereby clearly indicated how few phenomena in the living cell can be, as yet, fully accounted for by laws of physical chemistry. One may think of the example of urinary secretion. Descriptive physiology has shown that it consists of filtration across the glomerular membrane and selective reabsorption in the tubuli. Only the glomerular function is understood from the laws of diffusion and osmosis, whereas the second remains a complete physico-chemical mystery insofar as no model has been devised to imitate either selectivity or any mechanism to utilize the energy released by the chemical process of respiration to those specific tasks.

This book is particularly adapted for showing which biological events can be explained "from below," and which cannot as yet. Although there are a few remarkable achievements of the first kind, there are disappointingly more of the second. However, there is a distinct progress in some regions, while, on the other hand, there is even a retrogression in others. Thus, the permeability of the membrane of erythrocytes seemed to be rather well understood. It was then found that there are great and unexplainable differences according to the animal species on comparing the relative permeability of various simple nonelectrolytes.

The first half of the book is composed of a chapter by Hitchcock on selected treatises of classical physical chemistry, and on large molecules by Bateman, a chapter written with wide scope and embracing knowledge of this highly complex subject. The second, larger moiety of the book, deals with living matter itself. Höber, after a short, ex-

cellent introduction, deals with the surface of the cytoplasm and permeability problem. The same author, in another chapter, deals with the influence of extracellular agents on cellular activity. Then follows a short, concise chapter on the chemistry of respiration by Goddard. Furthermore, Fenn writes a chapter on contractibility abundant with descriptive material and clearly showing to what a tiny extent the facts can be subordinated to known laws of physical chemistry, due to the embryonic state of our knowledge of the complex nature of the physico-chemical systems involved. The final chapter, by Höber again, deals with "passive penetration and active transfer in tissues," the latter meaning all kinds of excretions and secretions occurring in living cells, utilizing the energy set free in metabolism, without any acceptable picture being available as to the mechanism by which chemical energy is exploited for specific work instead of being dissipated.

The reader will have understood that all the deficiencies mentioned are not those of the book but those of our present knowledge. The aim of the book is to point out both the achievements and the gaps. It fulfils the task splendidly. No student of physiology "from below" should miss the inspiration it affords. He will use it as a road-sign on his thorny path.

L. MICHAELIS, New York, N. Y.

**Virus as Organism.** Evolutionary and Ecological Aspects of Some Human Virus Diseases. By FRANK MACFARLANE BURNET. (134 pages.) Harvard University Press, Cambridge, Massachusetts. (1945.)

For the few years following the isolation of the tobacco mosaic virus the accepted concept among many biologists and biochemists working in the field of virus research was that viruses are chemical entities, specifically nucleoproteins, which arise in the cell as a consequence of some peculiar circumstance. The display of ingenuity in devising hypotheses of production *de novo* has, of course, been encountered before. This concept, however, has been strengthened by the research coming out of the chemical laboratories, inasmuch as "molecular weights," etc. have been reported for them, and some of them have been obtained in the crystalline form. These substances have lent themselves to manipulation in the laboratory as if they were chemical entities, and they have been so manipulated. There has been a tendency among biochemists working with the viruses to discuss the viruses in their published work as if they were indeed chemical entities. On the other hand, these workers have behaved in conducting their experimental work as though they believed they were dealing with infectious organisms. That is, quite apart from the treatment of endemic and epidemic virus infections by various agencies, research workers have inoculated their animal or plants with the respective viruses they were working with, with the full expectation that the self same virus would be reproduced. In the isolation work, Koch's rules of proof have been the criteria which have served as the guide to the several manipulations the plant or animal materials have been subjected to. Not only that, the control animals and plants have been isolated in order to avoid infection.

I have been conscious of an uncomfortable feeling among virus workers regarding the concept which has been referred to. In some cases the concept has been obviously abhorrent, but the presumptive evidence from the chemical laboratories regarding the chemical nature of the viruses has been strong. Many discussions in seminars,

scientific meetings, hotel rooms, and in print have revolved around this subject. It is doubtful that the intangible phenomena called life have ever been subjected to a more careful and searching scrutiny than they have been by the research workers with the viruses during the past decade. The arguments and beliefs, each colored by the particular prejudices of its author, have been many and varied.

The alternate concept regarding the nature of the viruses is that they are organisms, and a corollary to that concept is that they are the degenerate descendants of larger pathogenic organisms. This is Dr. Burnet's thesis, and he has marshalled his arguments in its support in a most masterful manner. Indeed, he has gained a convert and a disciple. It seems unlikely that in the last analysis one may avoid the conclusion that viruses are organisms. It is with the earnestness of a new convert that this book is recommended, in particular to the biochemists who have become involved in virus research.

Dr. Burnet has restricted his discussion to a few of the human viruses, namely, Herpes simplex, poliomyelitis, psittacosis, and related infections, smallpox, alastrin, vaccinia, yellow fever, and influenza. These viruses are considered from the point of view of their reproduction, their survival, their variation, and their evolution, and from the point of view of the reaction of the host to the virus infection. A long evolutionary history is postulated, although the impossibility of giving a precise account of the evolution of any of the viruses is recognized. Herpes simplex, for example, is considered to have evolved with man through his mammalian history. Psittacosis has been adapted to the Psittacidae since time immemorial. Its impact on man is a current event, and it cannot be regarded, strictly speaking, as a human virus. Yellow fever is and has been endemic among animals in the tropics, having evolved through the countless centuries to a form of life compatible to itself and to its "natural" hosts. *Aedes aegypti* is only one of them. Yellow fever infections in man are incidental in the history and evolution of the virus. The conflict between man and poliomyelitis is on, and the success the virus will have in establishing itself as a typical human virus, from an ecological point of view, will depend on the evolution in both man and the virus of forms mutually compatible and where the disease will present to man problems no more serious than those presented by Herpes simplex.

It is implied, but not specifically stated, that perhaps plant life can and may serve as a reservoir of viruses which may infect animals in much the manner that the Psittacidae serve as a reservoir of the psittacosis virus or that rodents may possibly serve as a source of poliomyelitis. The converse may also occur. Granted this possibility, then are the viriferous leaf hoppers, *Eutettix tenellus*, infected with the curly top virus of sugar beets? In these instances of insect transmitted virus diseases of plants do we have to deal with viruses which infect both plants and animals? These questions and many others are provoked by Dr. Burnet in his discussion. Indeed if his arguments are valid, we may dispense with the notion of specificity in the transmission of virus diseases by insects.

The temptation is strong to include herewith a synopsis of Dr. Burnet's arguments and to present a critical consideration of them. By doing so, however, one would be guilty of robbing the prospective reader of the fun he will have in pursuing the contents of this small book.

VERNON L. FRAMPTON, Austin, Texas

**Studies in Biophysics: The Critical Temperature of Serum (56°).** By LECOMTE DU NOÛY, D.Sc. Reinhold Publishing Corp., New York 1915. 185 pages. Price \$3.50.

The author admits that for a long time he believed that the majority of the proteins of serum existed in the shape of a large super-molecule, which he refers to as a "serum molecule." He states, however, that the work from Svedberg's laboratory indicates, on the contrary, that there are present in serum at least four distinct species of molecule. He states that the knowledge that the proteins—or lipo-proteins—of serum are molecularly dispersed makes it possible to introduce into biology "the rich and fertile idea of monomolecular oriented dispersion" which Dr. du Noüy says he has upheld since 1923.

In spite of this preliminary statement, it is the impression of this reviewer that Dr. du Noüy has continued to treat serum as if it were a single pure substance, such as copper or mercury, for in the present book we are presented with a summary of a great wealth of measurements, most of them of a physical or physical-chemical nature, on "serum" of one sort or another. No information is given as to the proteins present in these sera and their relative amounts in the samples used.

Figures are given for various properties of serum: the viscosity, the rotatory power and rotatory dispersion, the optical density and light scattering power, coagulation, sedimentation, electric conductivity, hydrogen ion concentration, fixation of ether, surface tension, and ultraviolet absorption. The effect of various temperatures for various lengths of time on these properties is also studied. If accompanied by chemical information about the samples studied, some of these measurements might have been valuable. The author makes some statements which seem somewhat doubtful to the present reviewer. On p. 95 it is stated that the volume of the molecules increases only when the temperature has reached about 55°. On p. 97 the author mentions the "fact" that below 54° serum is practically unalterable, no matter what the duration of the heating. The reviewer, having had occasion over a period of 20 years to try to preserve serum with as little alteration as possible, feels especially keenly about the latter statement.

From the observation that ten of the phenomena studied by him begin to be evident at a temperature of 56°, the author considers that this temperature is of particular significance. However justifiable this conclusion may be, the further step in reasoning, namely that therefore the destruction of alexin (complement), which also has a critical temperature in this neighborhood, is related to the structural and chemical modifications determined by heat in the "serum molecule" as a whole, seems a bit doubtful. It also seems too bad that the author does not seem to know of the brilliant work of Pillemer, Ecker, and others which demonstrates that complement is not a chemical entity, but consists of a number of separable components

WILLIAM C. BOYD, Boston, Mass.

**Ultracentrifugal Studies on Serum and Serum Fractions.** By KAI O. PEDERSEN. 178 pp. Printed in Sweden by Almqvist and Wiksells Boktryckeri AB, Uppsala, 1945. Agent for the U. S., G. E. Stechert and Co., New York, N. Y. Price \$2.50.

This thesis represents the result of prolonged studies carried out by the author at Uppsala in the years 1939 to 1944; it has grown from years of earlier work, much of

which has been reported in the great monograph of Svedberg and Pedersen on "The Ultracentrifuge."

Two major advances in our understanding of serum and plasma proteins are recorded here. One is the clarification of the nature of the "X-protein" of human serum, originally described by McFarlane, whose observations, however, left the nature of this substance very much in doubt. The clue to its distinctive character was obtained through a chance observation of Pedersen's, systematically followed up, which revealed an extraordinary decrease in the sedimentation rate of this component with increasing density of the solvent. Indeed, in solvents of density  $> 1.04$  the direction of sedimentation is reversed, showing that this is the approximate density of the protein itself. Since the density of most dry proteins is near 1.33, it is clear that the composition of the X-protein must be very unusual; and there is good reason to believe that it contains a very high proportion of lipids. Electrophoretically it appears to belong among the  $\beta$ -globulins. Pedersen believes that the X-protein "is a reversibly dissociable compound formed by albumin, globulin and some lipids with a total particle weight of the order of magnitude  $1 \times 10^6$ " (p. 32). Detailed evidence for this view - which is not to be regarded as completely established in all respects—is given in the report of numerous experiments. It is interesting to note that only human serum, among all the animal species yet studied, appears to contain appreciable quantities of X-protein.

The second major contribution of these studies is the characterization of a distinctive globulin, fetuin, which is a predominant component in the globulin of serum from cow's fetus, and is present in large amounts in the serum of newly born calves. It is also found in large quantities in the sera of the foal and of sheep's fetus, but it is practically nonexistent in the sera of adult animals. The molecular weight of fetuin is near 50,000, the sedimentation constant ( $s_{20}$ ) near 3.2 S. The asymmetry factor (1.6-1.8) indicates that the form of the molecule deviates markedly from the spherical. The identification and characterization of this protein represents a major advance in our knowledge of serum.

A large portion of the thesis is devoted to a detailed report of a long series of fractionation studies on the sera of man and various animals, involving salting out with ammonium sulfate at progressively increasing concentrations, pH being also varied between 5 and 7. Ultracentrifugal studies on the separated fractions are given in great detail; in a few cases electrophoretic analyses are reported also. Further separation of euglobulins by dialysis of some of the fractions was also carried out. From the point of view of the author's primary aim—the separation of individual components, as judged by the criterion of uniform sedimentation constants—the results were disappointing. Almost every fraction obtained proved to be a mixture, in spite of careful and systematic variation of salt concentration, pH, and the order of the different steps in fractionation, in a long series of carefully planned experiments. The data here reported, however, may serve as a valuable guide which will save much labor to future experimenters in this field. In short, this thesis is an indispensable work for all investigators seriously concerned with the study of the plasma proteins.

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# The Tryptophane Activity of Various Compounds for *L. arabinosus* and their Influence on the Determination of Tryptophane in Natural Materials\*

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Received February 18, 1946

## INTRODUCTION

The wide use of *L. arabinosus* for quantitative estimation of vitamins and amino acids depends upon its relatively specific requirements for these nutrilites. Nevertheless, the requirements for a given factor are not always rigidly specific, and interest has developed, for example, in the activity of compounds related to tryptophane when tested in tryptophane-free media. It has been shown that *l*-tryptophane is required by *L. arabinosus*, and that indole and anthranilic acid will produce growth of this organism equivalent to 30–90% of that obtained with *l*-tryptophane when compared on a molar basis (1, 2, 3). Several other compounds related to tryptophane were also tested for their tryptophane activity but were found to be inactive (2). The basal media used in these studies contained amino acids in the form of hydrolyzed casein and cystine. Tatum and coworkers (4, 5, 6) have studied the biosynthesis of tryptophane from indole with *Neurospora* mutants and have shown that serine is necessary for this synthesis.

Since media have been devised for *L. arabinosus* in which the amino acids are supplied in pure form (7, 8, 9) the apparent tryptophane activity of indole has now been measured in the presence and absence of serine when both the amino acid medium (9) and the cheaper but cruder casein hydrolyzate medium (10) were used. Xanthurenic acid,

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indoleacrylic acid, anthranilic acid and aniline were also tested for possible tryptophane activity and the possible interference of indole in the determination of tryptophane in rat urine has been investigated in some detail.

### EXPERIMENTAL

The microbiological techniques employed in this study with *L. arabinosus* are identical with those reported previously (9, 10). The *l*-tryptophane standard curve was obtained over a range of 0–25  $\gamma$ /tube and the acid production was determined after incubation at 37°C. for 72 hours. All tubes were adjusted with distilled water to a final volume of 10 cc.

Stock solutions of the compounds tested were prepared as follows: Indole was dissolved in water and made up to a final concentration of 100  $\gamma$ /cc. Anthranilic acid solutions were also prepared at this concentration and neutralized prior to addition to the medium. Xanthurenic acid and indoleacrylic acid<sup>1</sup> were dissolved in water with the aid of NaOH, neutralized and diluted to a final concentration of 200  $\gamma$  and 400  $\gamma$ /cc. respectively. Suitable dilutions were made from these solutions for testing apparent tryptophane activity. All solutions were prepared the same day as the tests were performed. Indole solutions were found to deteriorate when stored for 1 week.

#### *Influence of Serine on the Tryptophane Activity of Indole*

A previous study (9) showed that serine was not essential for the growth of *L. arabinosus* in media containing tryptophane. This observation has now been confirmed. When 1 mg. of *dl*-serine was added per tube of the amino acid medium or of the medium containing the casein hydrolyzate, the recoveries of *l*-tryptophane were 100–102% and 95–100%, respectively. In other words, serine did not alter the growth response of the organism. But when the tryptophane in the synthetic medium was replaced by indole, the growth response due to a given amount of indole was found to increase with increasing levels of serine. However, no increase in activity was observed when serine was added to the casein hydrolyzate medium presumably because optimal amounts of serine were already present in the hydrolyzate. Separate studies revealed that 1 mg. of *dl*-serine per tube was optimal for maximum indole activity with the amino acid medium. The

<sup>1</sup> Synthesized by Dr. J. A. Miller.

activity increased in the range of 50  $\gamma$  to 1 mg. of serine per tube with no further increase thereafter. One mg. of serine was used in all subsequent work.

The activity of indole was then tested at levels ranging from 2.5 to 60  $\gamma$  per tube in the presence and absence of serine on both media and compared to that of tryptophane on a molar basis. Three separate assays were made with triplicate tubes at each level of indole tested.

Indole alone stimulated the growth of *L. arabinosus* on both the synthetic medium (Fig. 1, Curve E) and on the casein hydrolyzate

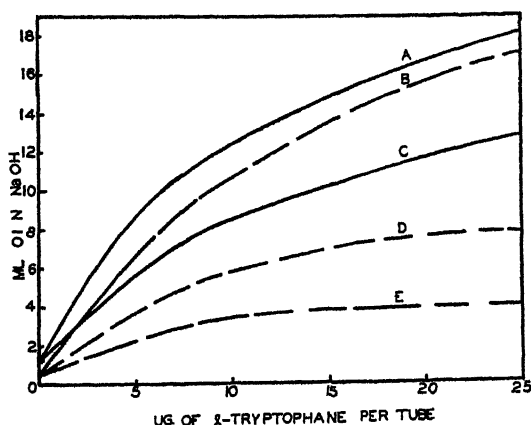


FIG. 1

Standard Curves Obtained for Indole and Tryptophane after Incubating *L. arabinosus* for 72 Hours at 37°C. The amounts of indole tested are expressed as tryptophane on a molar basis.

Curve A *L*-Tryptophane standard curve obtained with the casein hydrolyzate medium.

Curve B *L*-Tryptophane standard curve obtained with the purified amino acid medium.

Curve C Indole ( $\pm$  serine) with the casein hydrolyzate medium.

Curve D Indole + serine with the amino acid medium.

Curve E Indole (no serine added) with the amino acid medium.

medium (Curve C). Tryptophane stimulated growth to a greater extent than indole, and the amount of growth in the presence of the amino acid was essentially the same on the synthetic and crude media (Curves B and A, respectively). When serine was added to the synthetic medium, growth due to indole was enhanced (Curve D as

compared to E) but it never reached that observed when indole was added to the casein hydrolyzate (Curve C). Since growth on the synthetic medium plus indole and serine (Curve D) never equalled that on the hydrolyzate plus indole (Curve C), it appears that other factors in the casein hydrolyzate contribute to the apparent tryptophane activity.

TABLE I

*Tryptophane Activity of Various Compounds for L. arabinosus in the Presence and Absence of Tryptophane<sup>1</sup>*

(Values expressed as percentage activity of tryptophane on a molar basis)

Amount tested ( $\gamma$ /tube)	Indole*		Indole**		Anthranilic acid		Xanthurenic acid		Indoleacrylic acid	
	+ trypto.	- trypto.	+ trypto.	- trypto.	+ trypto.	- trypto.	+ trypto.	- trypto.	+ trypto.	- trypto.
2.5	42	71	7.3	45	8.4	42				
5.0	27	64	8.0	38	7.8	32		0		
7.5	25	58	5.9	32	—	28				
10.0	22	52		29	9.6	23		0		
12.5		51		25	—	23				
15.0		47		24	—	21		0		
20		44		20	6.3	20	0	0		
40		32		12	6.0	19	0		0	0
60		14		10			0	0	0	0
100							.2	.2	<5	0
150							.1		<5	.5-1
200							.1	.2	<5	.5-1
300							.1		<5	.5-1
400							.1	.1	<5	.5-1
800								.1	-5	0
1000						<1			-45	0
2000									-90	0

\* Tested with casein hydrolyzate medium.

\*\* Tested with synthetic amino acid medium + serine.

<sup>1</sup> 5 $\gamma$ /tube of indole were tested at the different levels of tryptophane indicated. For the other compounds different levels of the compounds were tested in the presence of 4 $\gamma$ /tube of tryptophane.

### *Activity of Other Related Compounds*

The activity of anthranilic acid was tested at levels ranging from 2.5-1000  $\gamma$  per tube. Xanthurenic acid and indoleacrylic acid were

tested at levels ranging from 5–800  $\gamma$  and 60–2000  $\gamma$  per tube, respectively. The activity of indole (both media), xanthurenic acid, anthranilic acid and indoleacrylic acid was also determined in the presence of tryptophane. In the case of indole, a constant amount was added and the level of tryptophane was varied; and for xanthurenic acid, anthranilic acid and indoleacrylic acid, 4  $\gamma$  per tube of tryptophane were added in conjunction with various levels of the test compounds. The results obtained for these experiments are given in Table I.

At levels of 2.5–40  $\gamma$  per tube, anthranilic acid was found to be from 20–40% as active as tryptophane on a molar basis while xanthurenic acid and indoleacrylic acid were essentially inactive. The activity of both indole and anthranilic acid was greatly reduced in the presence of tryptophane. High amounts of indoleacrylic acid (800–2000  $\gamma$  per tube) depressed growth progressively both in the presence and absence of added tryptophane.

In Table II, the results obtained with compounds tested for tryptophane activity with *L. arabinosus* in the present work and those from the literature are summarized.

TABLE II

*Summary of the Tryptophane Activity of Various Compounds for L. arabinosus*

Compound	Level tested ( $\gamma$ /tube)	Per cent activity (molar basis)	Reference
<i>l</i> -Tryptophane		100	
<i>dl</i> -Tryptophane		50	2, 12, 13
Indole	2–60	90–14	1, 2, 3, 13, present work
Indole-3-acetic acid	100	0	2
$\beta$ -(Indole-3)-propionic acid	100	0	2
$\gamma$ -(Indole-3)- <i>n</i> -butyric acid	100	0	2
Tryptamine hydrochloride	100	0	2
Anthranilic acid	2–40	50–19	1, 2, 3, present work
Xanthurenic acid	5–800	<1	present work
Kynurenic acid	100	0	2
Indoleacrylic acid	40–400	<1	present work
Aniline	5–3000	0	present work
Skatole	100	0	2
<i>p</i> -Aminobenzoic acid	1000	0	1
Orthanilic acid	10,000	0	1
3-Indolealdehyde	not given	0	13

*Effect of Indole on the Determination of Tryptophane  
in Natural Materials*

The preceding results might imply that *L. arabinosus* is not suitable for the quantitative determination of tryptophane in natural materials that also contain indole. However, Greene and Black (2) have shown that indole can be extracted with ether prior to analyzing for tryptophane and we have demonstrated that the apparent tryptophane content of human urine determined with *L. arabinosus* is essentially the same before and after extraction with ether (10).

The absence of indole in natural materials might also be indicated through the use of both *L. arabinosus* and either *S. faecalis* or *Leuconostoc mesenteroides* as test organisms; the latter organisms are unable to utilize indole to replace tryptophane (1, 12). Accordingly the tryptophane content of rat urine was determined with *S. faecalis* with the medium originally devised for the determination of threonine (11) (tryptophane was omitted from the basal medium and *dl*-threonine added at a level of 2 mg. per tube). Urine was collected from rats which had received adequate purified rations, the samples were prepared for assay as described previously (10), and simultaneous assays for tryptophane were carried out with both *S. faecalis* and *L. arabinosus*.

TABLE III  
*A Comparison of Values for the l-Tryptophane Content of Rat Urine  
Determined by Two Methods of Assay*

Sample No.	$\gamma$ of <i>l</i> -tryptophane per cc. of diluted urine ( <i>L. arabinosus</i> )	$\gamma$ of <i>l</i> -tryptophane per cc. of diluted urine ( <i>S. faecalis</i> )
1	0.40	0.41
2	1.47	1.33
3	0.28	0.36
4	1.34	1.19
5	0.85	0.83
6	0.39	0.43
7	2.75	2.90
8	3.20	3.40

The results (Table 3) show that similar values for tryptophane were obtained with both organisms for samples that varied in tryptophane over an 8-fold range of concentrations. These results thus substantiated the earlier observation that indole is not ordinarily

present in urine in amounts sufficient to interfere with the determination of tryptophane by *L. arabinosus*. Nevertheless, it should be possible to determine indole quantitatively with the two types of organisms. The total tryptophane activity determined with *L. arabinosus* minus the amount found with *S. faecalis* would give the activity due to indole in the sample. From data similar to those in Table I, the amount of indole accounting for this activity could be calculated.

### DISCUSSION

The present work suggests that the mechanism by which indole is utilized in replacing tryptophane for *L. arabinosus* is similar to that for the *Neurospora* mutants. The means by which anthranilic acid supplies apparent tryptophane activity is still obscure, although the presence of the amino group *o*- to the carboxyl group is essential, since *m*- and *p*-amino benzoic acid (1) and aniline possess no tryptophane activity. The extremely low activity observed for xanthurenic acid and indoleacrylic acid may be due to impurities rather than to the substances themselves.

Although most of the microorganisms thus far tested are highly specific in their amino acid requirements, the high activity of indole and anthranilic acid in replacing tryptophane for *L. arabinosus* is an exception. Fortunately, the means of detecting the interference of such compounds with the determination of tryptophane in natural materials with *L. arabinosus* are rapid and precise.

### SUMMARY

(1) Indole, anthranilic acid, xanthurenic acid, indoleacrylic acid and aniline were added to media free from tryptophane, and the production of acid by *L. arabinosus* measured after a 72 hour incubation period. In agreement with others, indole and anthranilic acid were found to increase the growth of this organism. Xanthurenic acid, indoleacrylic acid and aniline were virtually inactive.

(2) The activity of indole was increased by the addition of serine to a medium low in this amino acid. When a casein hydrolyzate was used, no increase in the activity of indole was noted when serine was added.

(3) The apparent tryptophane activity of indole (casein hydrolyzate medium) decreased from 70–14% as the concentration of indole was increased (molar basis) from 2.5 to 60  $\gamma$ /tube. The activity of indole

in the presence of tryptophane was much lower. The activity of anthranilic acid likewise was very low in the presence of tryptophane.

(4) The same values for *l*-tryptophane in rat urine were obtained whether *L. arabinosus* or *S. faecalis* was used as the test organism. Since the latter cannot utilize indole to replace tryptophane, it is apparent that urine does not contain sufficient indole to interfere with the tryptophane assay.

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# Factors Influencing the Maintenance of Blood Concentrations of Sulfonamides

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## INTRODUCTION

It is well known that, in order to maintain therapeutic concentrations in the blood, sulfonamides must be administered several times a day. Therefore, it is not surprising that interest was expressed in sulfadiazine and later in sulfamerazine when blood concentrations of these drugs were found to be maintained longer than with previous drugs. In a study of sulfaquinoxaline<sup>1</sup> conducted in this laboratory (Seeler, Mushett, Graessle and Silber, 1944), it was found that therapeutically effective concentrations persisted for several days after a single dose.

Several investigators (Gilligan, 1943; Davis, 1943; Fisher, Troast, Waterhouse and Shannon, 1943) have noted a relationship between the distribution of sulfonamide drugs in body fluids and their protein-binding capacity. Since the amount of drug in the circulation is only a small fraction of the total dose administered, it seemed possible that part of the dose might be stored or retained in the body and thus provide a relatively prolonged supply of drug to replace that removed from the circulation. In the present study, an attempt has been made to evaluate various factors which affect the maintenance of therapeutic blood concentrations of sulfonamides, with particular attention to absorption and excretion after oral and intravenous administration to rats.

<sup>1</sup> Synthesized by Weijlard, J., Tishler, M., and Erickson, A. E., *J. Am. Chem. Soc.* **66**, 1957, 1944.



## METHODS

Single doses of 200 mg./kilo were administered either orally or intravenously to adult white rats (150–250 g. each) of the Carworth strain. Oral doses were given in 2% aqueous suspension, intravenous doses in 4% solution. Except where noted, rats were fasted overnight and fed *ad libitum* 2 to 3 hours after dosing. Drug concentrations were determined by the method of Bratton and Marshall (1939) in blood, plasma and urine by direct assay, in tissue after homogenization by means of a glass tissue grinder,<sup>2</sup> and in feces and gastro-intestinal tracts after heating to boiling in alkaline solution. Blanks were included and corrections made for color or turbidity of the samples. In experiments with acetylsulfaquinoxaline, samples were first hydrolyzed at 100°C. with 0.2 *N* HCl for 1 hour. Paraffined metabolism cages were washed down thoroughly with water for determination of urinary excretion.

## RESULTS

The first section of this study was concerned with the fate of the drugs in the body and included determination of the concentrations in blood, plasma and liver, the gastro-intestinal content, and urinary and fecal excretion. After oral or intravenous administration of 200 mg. of sulfaquinoxaline (SQ), sulfamerazine (SM) or sulfadiazine (SD) per kilo body weight to fasted rats, analyses were performed as shown in Table I.

Although the initial plasma concentrations were similar, concentrations of SQ were 2 to 8 times those of SM or SD 24 and 48 hours after oral or intravenous administration. Concomitant assay of the intestinal contents revealed 2 to 4 times as much SQ, indicating a correlation between plasma concentration and the total amount of drug in the gastro-intestinal tract. Similarly, assay of the gastro-intestinal contents for SM after 24 hours revealed twice the amount of SD found at the same time, and this was correlated with a higher plasma concentration of SM.

The studies of the fate of the drugs after intravenous injection, therefore, assumed added importance because it was felt that any effect of drug in the gastro-intestinal tract might be minimized by this mode of administration. However, the plasma concentrations of SQ were again

<sup>2</sup> Scientific Glass Apparatus Co., Bloomfield, N. J.

distinctly superior to those of SD and SM, and assays of the contents of the gastro-intestinal tract showed that a correlation still existed between plasma concentration and intestinal content of drug. In these experiments the correlation was obviously effected by excretion into the intestinal tract. The extent of this excretion was shown by the

TABLE I

*Plasma Concentrations,\* Gastro-Intestinal Content and Excretion of Sulfaguanidine, Sulfamerazine and Sulfadiazine in Rats. (G.I. = gastro-intestinal tract; % = % of dose)*

After oral administration of 200 mg./kilo (averages, 4 rats each)

Time hours	Sulfaguanidine				Sulfamerazine				Sulfadiazine			
	Plasma mg. per cent	G.I. per cent	Urine per cent	Feces per cent	Plasma mg. per cent	G.I. per cent	Urine per cent	Feces per cent	Plasma mg. per cent	G.I. per cent	Urine per cent	Feces per cent
4	16.2	74.0			17.8	68.0			16.1	75.0		
8	14.7	40.0			16.8	43.0			9.4	65.0		
24	10.5	20.0	13.7	30.8	5.6	9.5	46.0	35.0	3.2	4.4	40.0	36.0
48	8.6	9.5	22.6	42.5	1.0	2.3	52.1	38.0	1.4	2.3	42.1	39.5

After intravenous administration of 200 mg. per kilo (averages, 2 rats each)

2	35.0	8.8			32.2	7.4			34.6	10.4		
4	35.0	12.3			25.0	9.5			31.4	13.0		
8	30.6	22.0			16.6	12.7			15.6	11.7		
24	19.0	14.0	24.6	12.3	4.3	2.9	39.0	13.7	4.4	2.9	40.0	8.0
48	8.2	5.8	30.3	23.8	0.8	1.1	41.6	21.2	2.6	1.4	42.1	11.5
72	2.5	2.6			0.5	0.7			1.8	1.0		

\* Blood concentrations averaged 74% of the plasma concentrations and on a mg./g. basis, liver concentrations averaged 45% of the plasma concentrations with no significant differences between drugs, so these have been omitted from the table.

finding of 22% of the intravenous dose of SQ in the gastro-intestinal tract after 8 hours and a 48 hour fecal excretion of 24%. After oral administration the urinary excretion of SQ was delayed. It was not until 4 to 5 days that the fraction of SQ excreted equalled that of SM or SD excreted in one day.

The correlation between plasma concentration and the amount of drug in the intestinal tract 24 and 48 hours after oral or intravenous administration indicated that retention of drug in the intestinal tract

might be primarily involved in the maintenance of blood concentrations. Two experiments have been performed to test this possibility.

In the first, 15 fasting rats were dosed orally with SQ, SM or SD and after 24 hours, the 3 sections of the gastro-intestinal tract were removed for assay as shown in Table II. Gastric retention of 30% of the SQ dose

TABLE II

*The Amount of Drug in the Gastro-Intestinal Tract 24 Hours after Oral Administration of 200 mg/kilo to Fasting Rats (averages of 5 rats each)*

Drug	Stomach per cent of dose	Small intestine per cent of dose	Large intestine per cent of dose
SQ	30.0	5.2	21.1
SM	negligible	2.0	14.0
SD	negligible	0.9	20.6

was observed after 24 hours whereas SM and SD had been almost completely eliminated from both the stomach and the small intestine. It is evident that this delay in emptying time of the gut must be involved in the maintenance of blood concentrations of sulfaquinoxaline since it affords a relatively continuous source of drug for absorption.

In the next experiment, 16 rats were dosed with SD after feeding and food was then withheld from half of them in an attempt to minimize loss of drug by decreasing the fecal output. Groups of 4 rats were sacrificed after 24 and 48 hours for comparison of gastro-intestinal drug content and blood concentrations. Those animals which were not fed after dosing had 2 to 3 times as much drug in their gastro-intestinal tracts and the blood concentrations were correspondingly 2 to 3 times higher than those of rats which were allowed to eat. Furthermore, the blood levels were substantially the same as those found in rats dosed with SQ (averages of 4 rats; 24 hours, 9.2 mg. % SQ, 9.6 mg. % SD; 48 hours, 4.7 mg. % SQ, 4.0 mg. % SD).

Since the intestinal assays in the previous experiments did not differentiate between drug which was passing along the intestinal tract with food and drug which had been excreted into the intestinal tract, these experiments yielded little information regarding the site or extent of absorption and excretion of the drugs. Therefore, 200 mg. of SQ, SM or SD/kilo (in 4% solution) were injected into the stomach, small intestine, large intestine or leg vein of anesthetized rats after the

three sections of the gastro-intestinal tract had been ligated. In this way absorption from, and excretion into, each section were studied. The results of assays made after 24 hours are summarized in Table III.

TABLE III

*Amount of Drug in Ligated Sections of the Gastro-Intestinal Tract 24 Hours after Injection of 200 mg. SQ, SM or SD/kilo*

Drug	Site of injection	Stomach per cent of dose	Small intestine per cent of dose	Large intestine per cent of dose	Blood concentration mg. per cent
SQ	Stomach	81.5	3.0	negligible	3.2
SM	Stomach	68.0	5.8	negligible	3.0
SD	Stomach	55.0	2.4	negligible	4.4
SQ	Small intestine	negligible	22.0	1.8	11.0
SM	Small intestine	negligible	16.5	1.0	3.0
SD	Small intestine	negligible	7.0	1.3	10.6
SQ	Large intestine	negligible	9.6	65.0	9.0
SM	Large intestine	negligible	12.2	40.4	5.2
SD	Large intestine	negligible	1.7	9.7	3.2
SQ	Vein	negligible	21.3	6.2	16.6
SM	Vein	negligible	24.0	2.0	3.6
SD	Vein	negligible	5.2	1.6	6.4

Sulfaquinoxaline was more slowly absorbed from each section of the gastro-intestinal tract than SM, and SM was in turn absorbed at a slower rate than SD. Absorption was best from the small intestine and slowest from the stomach. The large intestine was intermediate in absorptive capacity.

Excretion into the stomach was negligible in terms of fraction of the dose found after 24 hours. Significant amounts were excreted into the large intestine, but excretion into the small intestine was much more pronounced.

To determine the effect of modification of the sulfaquinoxaline molecule, the distribution of three derivatives<sup>3</sup> was investigated—the acetyl (*N*<sup>4</sup>-acetylsulfaquinoxaline), the hydroxy (2-sulfanilamido-3-hydroxyquinoxaline) and an amino derivative (2-sulfanilamido-6-

<sup>3</sup> Kindly furnished by Dr. K. Pfister and Dr. F. J. Wolf of the Merck Research Laboratories, Rahway, N. J.

aminoquinoxaline). The acetyl and hydroxy derivatives are of particular interest since they are metabolic products of sulfaquinoxaline (Scudi and Silber, 1944). Results after oral and intravenous administration are summarized in Table IV; for comparison with SQ, SM and SD see Table I.

TABLE IV

*Plasma Concentrations, Gastro-Intestinal Content and Excretion of Sulfaquinoxaline Derivatives in Rats*

After oral administration of 200 mg./kilo (averages 4 rats each)

Time hours	Hydroxysulfaquinoxaline				Aminosulfaquinoxaline				Acetylsulfaquinoxaline <sup>*</sup>			
	Plasma mg. per cent	G.I. per cent	Urine per cent	Feces per cent	Plasma mg per cent	G.I. per cent	Urine per cent	Feces per cent	Plasma mg. per cent	G.I. per cent	Urine per cent	Feces per cent
4	0.55	78.0			0.5	84.0			9.8	69.0		
24	1.2	17.0	2.3	50.0	0.4	9.3	3.7	78.0	2.2	3.0	32.0	25.0
48	0.5	8.0	4.8	58.0	0.4	3.1	6.2	81.0	1.3	1.4	36.9	25.0

After intravenous administration of 200 mg. per kilo (averages 2 rats each)

4	18.0	15.4			13.0	33.0			12.4	22.0		
24	17.4	23.0	25.0	5.3	1.2	7.2	20.8	10.4	5.2	2.0	61.7	8.0
48	4.7	8.0	34.7	6.4	0.6	4.2	26.6	36.4	2.3	1.5	63.5	11.5

\* After oral administration of acetylsulfaquinoxaline, 20-30% of the drug in the plasma, urine and feces was hydrolyzed to sulfaquinoxaline.

The small fraction of the oral dose of amino SQ or hydroxy SQ found in the urine, the large fraction in the feces and the low plasma concentrations showed that these derivatives were poorly absorbed. This may be correlated with their low solubility (Table V). On the other hand, the soluble acetyl derivative was absorbed and excreted rapidly. Like SQ, hydroxy SQ was retained in the gut after oral or intravenous administration. This resulted in prolonged blood concentrations after intravenous administration, but after oral administration poor absorption limited the blood concentrations to about 1 mg. %.

The rapid urinary excretion of acetyl SQ and slow excretion of SQ suggested that there might be a striking difference in binding by plasma protein. However, when binding was measured in rat plasma

by the procedure outlined by Davis (1943) and calculated by the method of Gilligan (1943), it was found that there was no significant difference between the binding of SQ and any of its derivatives. SQ was bound to the extent of 88.5%, amino SQ, 93%, hydroxy SQ, 92.5% and acetyl SQ, 93.5%. SM and SD were bound 74.5% and 62%, respectively.

TABLE V

*Drug Solubilities in M/15 Phosphate Buffer at 37°C., in mg. %<sup>a</sup>*

	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Sulfaquinoxaline	1.3	1.4	11.6	100.0
Hydroxysulfaquinoxaline	0.4	0.6	2.7	19.0
Aminosulfaquinoxaline	1.1	1.1	2.9	23.0
Acetylsulfaquinoxaline	7.4	30.0	267.0	850.0 <sup>††</sup>
Sulfamerazine	35.0	38.5	82.0	600.0
Sulfadiazine	12.0	15.4	76.0	580.0

<sup>a</sup> Equilibrium between solid and liquid phases was obtained within one week and the pH at equilibrium was measured with the glass electrode.

<sup>††</sup> pH 7.6.

## DISCUSSION

Two drugs which are bound by plasma protein to the extent of 93% (amino SQ and acetyl SQ) failed to maintain significant blood concentrations after either oral or intravenous administration. After oral administration of amino SQ, the significance of the 93% protein binding was superseded by the extreme insolubility of the drug which resulted in loss of a major fraction in the feces. When the drug was given intravenously, however, the rate of excretion became the controlling factor and the blood concentrations decreased rapidly, in spite of the high degree of plasma binding. Biliary excretion (Bettman and Spier, 1939, and Shay, Komarov, Siplet and Fels, 1944) was presumably enhanced, or reabsorption was limited, since 36% of the drug was found in the feces as compared with 6.4% for hydroxy SQ, which has approximately the same solubility.

The N<sup>4</sup>-acetyl derivative, on the other hand, is quite soluble and is quickly removed from the blood in spite of the high degree of protein binding in the plasma. The excretion of 32% in the urine within one day after the oral dose and 62% one day after the intravenous dose

shows that this drug is eliminated by the kidney more efficiently than SQ, hydroxy SQ or amino SQ, possibly in part by tubular secretion like N<sup>1</sup>-acetylsulfanilamide (Fisher, Troast, Waterhouse and Shannon, 1943).

The critical factor in the maintenance of SQ blood concentrations is the retention of drug in the gastro-intestinal tract which results from excretion into the intestine and delayed emptying time. Hydroxy SQ, which is less soluble than SQ, is poorly absorbed but, like SQ, is slowly eliminated from the gut. When poor absorption is eliminated as the limiting factor by intravenous administration, the blood concentrations are maintained almost as well as those of SQ, apparently as a result of accumulation of drug in the gut (23% of the dose after 24 hours, 8% after 48 hours). This seemingly contradictory result may be explained in one of the following ways: either the drug excreted into the intestine had been converted, at least in part, to a form which was absorbed more readily than hydroxy SQ, or the prolonged absorption of hydroxy SQ from the gut, although slight, was sufficient to compensate to a large degree for the loss in the urine.

The presence of food decreases blood concentrations by inhibiting absorption and increasing fecal loss of drug. Drug administered to fasting animals is more efficiently absorbed and retained in the gastro-intestinal tract longer, thereby both increasing and prolonging blood concentrations. By restricting feeding, fecal loss of SD has been minimized and blood concentrations have been maintained as long as those of SQ. Administration of sodium bicarbonate to rats receiving SQ has been found to aggravate the toxicity by increasing the concentration of drug in solution in the intestine, and thereby enhancing absorption.

Accumulation in the tissues would tend to retain drug in the body, but there has been little evidence to support this possibility. No accumulation in the livers of rats has been observed after single oral or intravenous doses. The concentration in the liver varied directly with the concentration in the blood. Furthermore, in monkeys dosed daily for one month with 100 mg. SQ per kilo, the concentration in the tissues (brain, heart, liver, muscle and spleen) ranged between 0.05 and 0.2 mg./g.

After comparing the prolongation of blood concentrations with the degree of protein binding of a large series of sulfonamides, Van Dyke, Tupikova, Chow and Walker (1945) have also concluded that a high

degree of protein binding does not alone prolong blood concentrations of sulfonamides.

### SUMMARY

1. Although sulfaquinoxaline was bound to the extent of 90% in rat plasma, compared with 75% for sulfamerazine, a more significant factor involved in prolongation of blood concentrations of the drug was the delay in emptying time of the gastro-intestinal tract. As much as 30% of an oral dose was found in the stomachs of fasting rats after 24 hours.

2. N<sup>4</sup>-acetylsulfaquinoxaline and 6-aminosulfaquinoxaline were bound in plasma to the same degree as sulfaquinoxaline, but did not maintain therapeutically significant blood concentrations after oral or intravenous administration.

3. Like sulfaquinoxaline, 3-hydroxysulfaquinoxaline was 90% bound in plasma, was excreted into the intestinal tract to a large extent and was slowly eliminated from the gut. However, it was poorly absorbed after oral administration, apparently due to its low solubility.

4. The concentration of sulfadiazine in the blood was maintained, as well as that of sulfaquinoxaline, when food was withheld after oral administration of the drug. This was a direct result of decreasing fecal loss of the drug, thereby retaining drug in the intestinal tract.

5. Absorption of sulfaquinoxaline, sulfamerazine, and sulfadiazine from 3 sections of the gut was compared by determining the fraction of a dose remaining in ligated sections 24 hours after injection. All were absorbed most from the small intestine and least from the stomach, with the large intestine intermediate in absorptive capacity. Sulfaquinoxaline (the least soluble) was the most slowly absorbed from all 3 sections. Excretion into the small intestine was approximately equal to urinary excretion. Excretion into the stomach was negligible, but significant amounts were found in the large intestine after intravenous dosage.

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# A Growth Factor for *Lactobacillus Gayoni* 8289 \*

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## INTRODUCTION

In a study of the growth requirements of over thirty species and strains of lactobacilli (1), it was found (2) that *Lactobacillus gayoni* 8289 failed to grow in a medium which permitted optimum growth of the other strains. On addition of yeast or liver extracts to the growth medium, *L. gayoni* 8289 grew abundantly. Undoubtedly these natural materials contain many unknown chemical compounds, and it appears evident that they supply a substance (or substances) required by this bacterium which was not found in any of the growth media used. This paper reports experiments which demonstrate the non-identity of this substance with known growth-promoting agents, and presents methods used in preliminary characterization and concentration of the factor. For convenience, it is referred to throughout as the Gayoni factor.

## EXPERIMENTAL

### *Organism*

*Lactobacillus gayoni* 8289, American Type Culture Collection, is kept on stab cultures in a medium containing 1% glucose, 1% Difco yeast extract, and 2% agar. Stabs are incubated at 37°C. until heavy growth is obtained (24 to 36 hours) and then kept in a refrigerator. Stock cultures are transferred every month, while stabs for daily use are prepared weekly.

Cultures for inocula are made by transferring cells from a stab to a sterile liquid medium suitable for growth. Such a medium is prepared by adding 5 mg. of a liver extract to 5 ml. of the basal medium (described later) and diluting to 10 ml. with water. The culture is then grown for 24 hours at 37°C.

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*Basal Medium*

The basal medium used throughout these experiments is given in Table I. It is a modified Snell and Wright medium (3, 4) containing, in addition, extracts of Difco yeast and peptone which have been freed of the Gayoni factor by adsorption on Norit and Lloyd's reagent. This medium supports luxuriant growth of *L. casei*, 12 ml. of 0.1 *N* acid being produced in the "blank" tubes after 72 hours. *L. gayoni*, by contrast, grows very poorly in this medium, but responds quantitatively when graded amounts of a source of the Gayoni factor are added, producing up to 20 ml. of 0.1 *N* acid in the presence of 2 mg. of Wilson's solubilized liver (Fraction L) or Lederle liver concentrate.<sup>1</sup>

TABLE I  
*Basal Medium for L. Gayoni*

Casein, vitamin free, H <sub>2</sub> SO <sub>4</sub> or HCl hydrolyzed.....	10	g.
Peptone, Norit adsorbed.....	5	g.
Yeast, Lloyd's reagent adsorbed.....	2	g.
Sodium acetate, anhydrous.....	40	g.
Glucose.....	40	g.
Cystine.....	200	mg.
Tryptophan.....	200	mg.
Adenine sulfate, guanine hydrochloride, uracil, thymine..... each	20	mg.
Riboflavin.....	400	γ
Thiamin, nicotinic acid, calcium pantothenate, pyridoxine hydrochloride, <i>p</i> -aminobenzoic acid..... each	200	γ
Biotin.....	0.8	γ
Folic acid*.....	0.25	γ
Salt solutions A and B (14)..... each	10	ml.
Distilled water to 1000 ml.; pH to 6.7		

\* Pure liver folic acid or a spinach concentrate (potency 7500) are used.

The preparation of the above extracts is carried out as follows: 16 g. of dried Difco yeast are dissolved in 400 ml. of distilled water, the pH is adjusted to 1 with concentrated HCl, and 16 g. of Lloyd's reagent are added. The mixture is shaken mechanically for one hour, filtered and the process repeated with a fresh 16 g. sample of Lloyd's reagent. The final solution is adjusted to pH 6.7 before using. It is preserved under toluene in the refrigerator. The final concentration of yeast is 40 mg. per ml. based on the original extract.

The same procedure is followed for Difco Bacto-Peptone, except that 20 g. of peptone in 400 ml. solution are adsorbed on 3 g. of Norit at pH 1.5. The final concentration of peptone is thus 50 mg. per ml.

<sup>1</sup> Lederle liver concentrate 1:15.6 is a hot water-soluble extract of fresh liver evaporated to dryness. Wilson liver fraction L is the 70% ethyl alcohol-insoluble fraction of whole liver solution which is redissolved in hot water and evaporated to dryness.

### Assay Procedure

The procedure followed is similar to microbiological assay of B vitamins. Samples are arranged in test tubes containing 5 ml. of the liquid medium adjusted to pH 6.7 (suitable for growth). A standard curve, which is included with each series of assays, contains 0, 0.25, 0.5, 0.5, 1.0, 1.0, 1.5, and 2.0 mg. of liver powder per tube (10 ml.). Samples to be assayed are usually tested at varying levels equivalent to 0.2–0.8 mg. of liver powder.

Tubes are cotton-plugged and sterilized by autoclaving for 15 minutes at 15 pounds pressure. After cooling, they are inoculated aseptically with 1 drop of a dilute inoculum prepared by adding 1 ml. of the 24-hour culture (previously described) to 10 ml. of sterile 0.9% NaCl solution. The tubes are incubated at 37°C. in a water bath. Growth is measured by turbidimetric readings after 36 to 45 hours by use of a suitable turbidimeter, or by titration of the lactic acid produced with 0.1 *N* NaOH after 68 to 72 hours. Comparative activities of samples are obtained by reference to the standard curve. (See Figs. 1 and 2.)

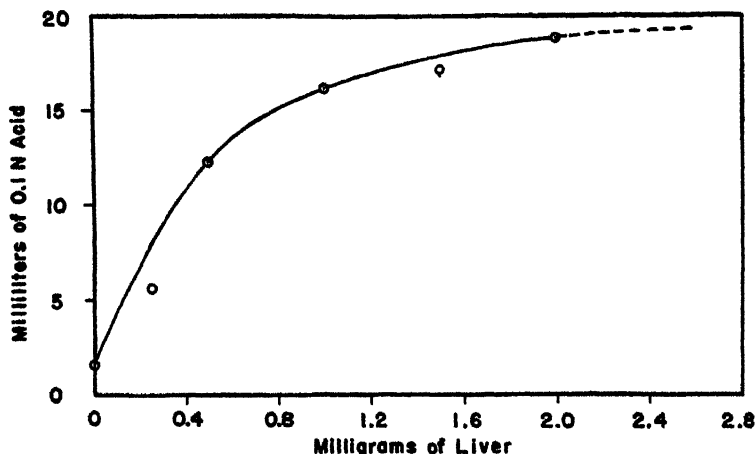


FIG. 1

Turbidimetric Response of *Lactobacillus gayoni* to the Gayoni Factor  
in Solubilized Liver (Fraction L)

Optical density equals log 100 minus log per cent transmission (equals  $2 - \log G$ )

### Response of *L. gayoni* to Known Compounds

Before intensive efforts were made to concentrate the Gayoni factor, a large number of growth-promoting agents were tested for activity with *L. gayoni*.

Growth in the basal medium could not be improved by altering the concentrations of the ingredients or by the addition of inositol, choline

and asparagine in varying amounts. Numerous nitrogenous substances were tested, since preliminary work suggested the presence of basic groups in the factor. All were inactive. These included: the 21 amino acids derived from proteins (alone or in combination, supplementing those present in the medium), *dl*-alanine (with and without pyridoxine (5)),  $\alpha$ -aminobutyric acid,  $\alpha$ -aminoisobutyric acid,  $\alpha$ -aminocaproic acid,  $\alpha$ -aminocaprylic acid,  $\alpha$ -amino- $\alpha$ -methylbutyric acid,  $\alpha$ -aminovaleric acid,  $\delta$ -aminovaleric acid,  $\epsilon$ -benzoylamino-caproic acid, glutamine, ornithine and phenylglycine. The tripeptide glutathione was

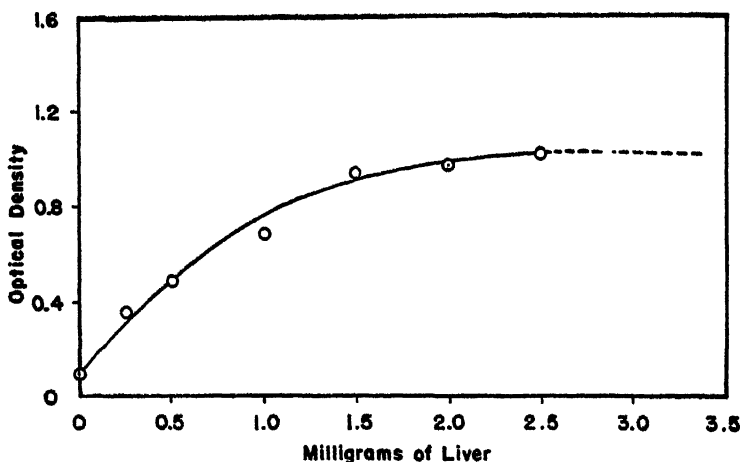


FIG. 2

Acid Produced by *Lactobacillus gayoni* in Response to the Gayoni Factor in Solubilized Liver (Fraction L)

tested, as were the following pyrimidines and purines: yeast nucleic acid (*per se* or various hydrolysis products), xanthine, hypoxanthine, 2-amino-4-diethylaminopyrimidine, 2-amino-4-hydroxy-5-methylpyrimidine, 4-amino-5, 6-dimethylpyrimidine, 2-aminopyrimidine, 6-aminopyrimidine, 2,4-diaminopyrimidine, 2,5-diethyl-6-aminopyrimidine, 5-ethyl-6-aminopyrimidine, 2-methyl-6-aminopyrimidine, 4-methyl-6-aminopyrimidine, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, 4-methyluracil, isocytosine and xanthopterin. Pyridine carboxylates which were ineffective were arecaine, arecoline, trigonelline, N-methylnicotinamide, and nicotinuric acid, as well as the growth

factors nicotinamide, coenzymes I and II (alone, together, or their acid or alkali hydrolysis products),  $\alpha$ -pyracin,  $\beta$ -pyracin,  $\beta$ -pyracin (free acid), pyridoxal, pyridoxamine, the acid or alkali hydrolysis products of folic acid (6), the *L. casei* yeast factor (7), crystalline vitamin B<sub>6</sub> (8), an orange peel factor (9) and pimelic acid.

Stimulation of growth by "streptogenin"-like material (10) has been observed for *L. gayoni*. If the yeast and peptone preparations are omitted from the basal medium, maximum growth and acid production are not obtained with the amounts of liver extract indicated in Figs. 1 and 2. The resulting curves are sigmoidal in shape, having a relatively low slope at the beginning and reaching only about three-fourths of the maximum produced in the presence of yeast and peptone. However, "streptogenin" or other similar material in yeast, peptone or trypsinized casein (11) cannot replace the Gayoni factor for growth, as is evidenced by the low readings in the blank tubes of Figs. 1 and 2.

#### *Sources of the Factor*

Table II shows relative potencies of the Gayoni factor in natural materials. All values are compared to Wilson's Liver Fraction L, which is the richest source of the factor yet found. Vegetables are seen to be relatively rich sources; cabbage contains almost half as much as fresh liver on a dry weight basis.

TABLE II  
*Sources of the Gayoni Factor*

Source	Method of Preparation	Relative Activity
Liver powder (Lederle or Wilson L.)	Water solution	1
Beef liver (fresh)	Papain and takadiastase (12)	0.15
Pork liver (fresh)	Papain and takadiastase	0.14
Pork liver (fresh)	Water hydrolyzed	0.017
Pork liver (fresh)	0.1 N H <sub>2</sub> SO <sub>4</sub> hydrolyzed	0.033
Hamburger	Papain and takadiastase	0.035
Peptone	Water solution	0.20
Yeast extract (Difco)	Water solution	0.83
Vitamin-free casein	1 N HCl hydrolyzed 1 hour	0.04-0.12
Vitamin-free casein	Trypsin hydrolyzed 2 days (11)	0.06-0.2
Dehydrated white potato	0.1 N acid + papain and takad.	0.1 -0.15
Dehydrated cabbage	0.1 N acid + papain and takad.	0.23-0.33
Dehydrated tomato	0.1 N acid + papain and takad.	0.14-0.22
Tomato juice	0.1 N acid hydrolyzed	0.042

The mode of extraction appears to have a marked effect upon the yield from fresh liver. Incubation with takadiastase and papain (12) results in nearly a ten-fold increase in activity over that obtained by hot water extraction.

### *Characterization Studies*

**Reactions of the Factor. Hydrolysis.** The Gayoni factor as found in liver powder is stable to digestion with takadiastase and papain at pH 4.5 (12) but is destroyed completely by autoclaving 15 minutes with 6 *N* H<sub>2</sub>SO<sub>4</sub>. It is only 50% stable to either 30-minute autoclaving with 0.5 *N* NaOH or 24-hour cold treatment with 1 *N* NaOH.

**Oxidation.** Excess cold neutral KMnO<sub>4</sub> or 6% H<sub>2</sub>O<sub>2</sub> destroys about 20% of Gayoni activity. The permanganate was added dropwise until excess was noted and the solution was filtered. For the peroxide treatment, 1 ml. of the 6% reagent was added to 5 ml. of liver solution (20 mg./ml.) and allowed to stand at room temperature for 30 minutes. Excess H<sub>2</sub>O<sub>2</sub> was steamed out.

**Bromination.** Excess bromine water was added dropwise to the liver solution. The solution was then boiled and filtered free of insoluble material. Only 40% of the original activity remained.

**Nitrous Acid Treatment.** Approximately 70% of the total activity is destroyed by reaction with nitrous acid. Eight-tenths g. of NaNO<sub>2</sub> and 1 ml. of glacial acetic acid were added to the liver solution and allowed to stand at room temperature for 24 hours. The solution was then carefully evaporated to dryness twice, redissolved and tested.

**Esterification.** The Gayoni factor is destroyed up to 60% when liver powder is treated with 1% fuming H<sub>2</sub>SO<sub>4</sub> or 0.45 *N* HCl in methanol. All hydrolysis attempts with KOH failed, however.

**Heat.** The Gayoni factor is stable to dry heat at 100°C. for a few minutes. However, maintenance of this temperature for one hour or more renders much of the liver powder insoluble in water and completely destroys its Gayoni activity.

**Precipitation Reactions. Mercury.** Either mercurous nitrate or mercuric acetate precipitates the factor almost completely. When the mercurous compound is decomposed with excess HCl, about one-third of the activity can be recovered. Fractional mercuric acetate precipitation according to the method of Warburg (13) was 98–100% complete, and recovery by H<sub>2</sub>S decomposition was as high as 70%.

About one-third recoverable activity remained with the precipitated  $\text{HgS}$  and could be separated from it by boiling in water.

**Lead.** Basic lead acetate (14) precipitated 80–90% of total Gayoni activity. Recovery by precipitation with  $\text{H}_2\text{S}$  and boiling with water was 85% complete.

**Silver.** Excess silver nitrate precipitated a large portion of the active factor, but only 30% of this amount was recoverable by boiling the residue with excess concentrated  $\text{HCl}$ .

**Phosphotungstic Acid.** Excess phosphotungstic acid precipitated 95% of the activity. Only 25% of this was recovered from the residue when barium hydroxide and sulfuric acid were used to decompose the precipitate and clear the filtrate.

**Flavianic Acid.** About two-thirds of the factor came down as a flavianate. The acid was added in excess and the filtrate cleared with barium hydroxide. Sulfuric acid was used to remove excess barium. Hot  $\text{Ba}(\text{OH})_2$  decomposed the residue to release 10% of the precipitated activity.

**Dialysis.** Twenty-four hour dialysis of the factor removed about 60% of the activity from the original solution. A liver solution of 20 mg./ml. in water was placed in a cellophane bag in a beaker and cold tap water was run into the beaker for the 24-hour period.

**Solubility-Fractionation.** *Immiscible Solvents.* Selective solubility of the Gayoni factor in immiscible solvents showed it to be exclusively soluble in water and not at all in the non-polar solvent. Solvents used were ether, *i*-butyl alcohol, *n*-amyl alcohol, *n*-butyl alcohol and *i*-amyl alcohol. The last two were used in a continuous Dakin extractor (15) run for 24 hours.

*Miscible Solvents.* Glacial acetic acid and 95% ethyl alcohol dissolved up to 50% of the factor. Dry acetone dissolved only about 10%. Water-solvent fractionations with ethyl alcohol and with acetone showed that the active component could be precipitated from water solution in varying amounts depending upon the volume-proportion of the organic liquid. Up to 83% could be successively fractionated as insoluble material from a 60–73–80% acetone solution.

**Adsorption-elution Characteristics.** *Charcoal.* The activated charcoals Norit, Norit A, and Darco G-60 adsorb the Gayoni factor readily in the acid pH range and slightly less easily at about pH 11. At pH 1 to 3, shaking with 20% charcoal by weight twice for 1 hour each time results in 90–95% adsorption. The activity can be eluted from basic



solution, the best results being obtained with ammonia. Exhaustive elution with hot 28%  $\text{NH}_4\text{OH}$  gives consistent yields of nearly 100% of total activity adsorbed.

*Fuller's Earths.* Lloyd's reagent and a variety of other fuller's earths adsorbed 85-90% of Gayoni activity at pH 1 and less at higher pH values tested up to 5.5. Equal weight of the adsorbent and liver powder were used. The same adsorption results when the powder is shaken once for 15 minutes as after two treatments for one hour each time. Cold 0.3 *N* or 0.5 *N*  $\text{NaOH}$  shaken with the reagent for a few minutes removes nearly all of the adsorbed activity.

*Alumina.* Dry aluminum oxide powder in a column adsorbs 90-95% activity at pH 3 or 5 and none at pH 1. Simple 15 minute shaking with 200% by weight of the alumina gave poorer results. Fairly satisfactory release in either case results with 20%  $\text{NH}_4\text{OH}$  elution.

*Other Adsorbents.* Other adsorbents which did not satisfactorily adsorb the Gayoni factor from liver powder at various pH values were Decalso, Florisil, Anex and Amberlite IR-1 or IR-4. The small amount of adsorbed material was, in a few instances, eluted completely with strong base.

### *Preparation of Concentrates*

On the basis of information obtained through characterization studies, a suitable method has been devised for concentrating the Gayoni factor from large quantities of Wilson's solubilized liver. It is described below, and is outlined in Fig. 3 for two 500 g. batches (=1,000,000 mgu.) of liver fraction L.

Since the Gayoni factor has not been purified, it is necessary to use arbitrary units to designate the weights and concentrations present. The degree of concentration is therefore given in terms of "potency" ( $\pi$ ), which is a measure of the activity of solid material at any step. All samples are compared to Wilson's liver fraction L, which is assigned a "potency" of 1. The measure of *total* activity is given in terms of "milligram units." One milligram unit (mgu.) is the amount of activity present in one mg. of liver fraction L.

Five hundred g. of liver fraction L were dissolved in about one liter of distilled water by careful stirring. The pH was adjusted to 2.8-3.0 with concentrated  $\text{HCl}$  and the solution transferred to a 12-liter round-bottom flask. Distilled water was added to make 10 liters of solution, 100 g. of Norit added and the mixture stirred mechanically for 45 minutes to 1 hour. At the end of this time, the solution was filtered

FIG. 3

## Scheme for Concentration of the Gayoni Factor

(Total initial activity = 1,000,000 Mgu.)

Batch 1		Batch 2	
20% Norit		20% Norit	
1st cake	2nd cake	1st cake	2nd cake
28% $\text{NH}_4\text{OH}$	Filtrate	28% $\text{NH}_4\text{OH}$	Filt.
250,000 Mgu. $\pi = 6.9$	75,000 Mgu.	100,000 Mgu. $\pi = 4.2$	70,000 Mgu.
Lloyd's ads., NaOH eluate		Lloyd's ads., NaOH eluate	
Filt.	Filt.	Filt.	Filt.
80,000 Mgu.	110,000 Mgu.	60,000 Mgu.	65,000 Mgu.
150,000 Mgu. $\pi = 10.7$		80,000 Mgu. $\pi = 8.2$	75,000 Mgu. $\pi = (8)$
50% Norit		50% Norit	
1st cake	2nd cake	1st cake	2nd cake
28% $\text{NH}_4\text{OH}$	28% $\text{NH}_4\text{OH}$	28% $\text{NH}_4\text{OH}$	28% $\text{NH}_4\text{OH}$
Filt.	Filt.	Filt.	Filt.
720,000 Mgu. $\pi = 41$	100,000 Mgu. $\pi = 20$	300,000 Mgu. $\pi = 37.5$	40,000 Mgu. $\pi = 11.8$

through Celite at about 50 pounds pressure. The Norit was washed while in the filter with 1 to 2 liters of water. The charcoal (with the Celite) was eluted immediately by boiling with 200 to 300 ml. of 28%  $\text{NH}_4\text{OH}$  and filtering. This elution was repeated until the filtrate showed very little color. The total volume of eluate was usually about 1 liter. In the meantime, the original filtrate from charcoal treatment was adsorbed on a fresh 100 g. batch of Norit and eluted in similar manner. The eluates were steamed separately to small volume to remove ammonia, 1 ml. of 1  $N$   $\text{NaOH}$  being added to aid its release. The pH of such solution after steaming was found to be approximately 6. The solutions were then made to convenient volume (usually 250 ml.) with distilled water and filtered free of any charcoal still present. Small aliquots were removed for dry weight determination and assay.

For subsequent Lloyd's adsorption, the remaining solution was adjusted to pH 1.5 with concentrated  $\text{HCl}$ . After the total weight of solids was obtained, an equal weight of Lloyd's reagent was added and the mixture shaken for 45 minutes. The Lloyd's reagent was removed by centrifuging and eluted with approximately 100 ml. of 0.5  $N$   $\text{NaOH}$ . The elution was repeated 3 to 5 times with 50-100 ml. portions of  $\text{NaOH}$  and all eluates combined. Solids were determined in the supernatants after adsorption and solids in the eluates were assumed by difference, since they contained large amounts of  $\text{NaOH}$ . Potencies calculated from these weights may, therefore, be somewhat unreliable.

Following this, identical Lloyd's eluates from two 500 g. batches were combined and adsorbed on 50% Norit at pH 3 twice as previously described. Again, the charcoal cakes were eluted separately with hot 28%  $\text{NH}_4\text{OH}$  and the solutions steamed to small volumes.

Using this procedure, the bulk of the factor was obtained as a concentrate of potency approximately 40, which is about 300 times the concentration in fresh liver. An overall apparent yield of 114% of the original activity was obtained in all fractions. This did not include an additional 50 to 60% which was lost in the filtrates from the various adsorption treatments. The apparent increase in activity which accompanies adsorption and elution has been observed frequently, both with Norit and Lloyd's reagent. It seems likely that these adsorbents may remove growth inhibitors from the liver, thus increasing the sensitivity of the organism to the Gayoni factor.

## DISCUSSION

The unique growth requirements of *L. gayoni* 8289 which were noted at the outset with respect to other lactic acid bacteria also apply when this organism is compared to other strains of *L. gayoni*. Thus, *L. gayoni* F20 exhibits luxuriant growth in the presence of alkali-treated yeast and peptone with no added B vitamins except riboflavin (1). Similarly, good growth may be obtained with *L. fermenti* 36 if thiamine is added

to the medium (1). The latter organism is apparently identical with *L. gayoni* 36 (16, 17, 18); in fact it may be possible (18, 19) that other strains of *L. gayoni* should be renamed as strains of *L. fermenti*.

A study of the properties of the Gayoni factor makes possible a preliminary classification of the substance and a comparison of it with other recently reported growth factors which have not as yet been fully identified. Several of these compounds were available for testing, and, as shown, gave no activity for *L. gayoni*.

The factor clearly shows acid properties in its adsorption in the low pH range and its precipitation with heavy metals, even though esterification experiments were not decisive. The extent of acidity appears small, however, since alumina adsorbs it nearly quantitatively at pH 3 but not at all at pH 1. Basic properties are suggested by its adsorption on Norit at pH 11, precipitation with flavianic and phosphotungstic acids and destruction by nitrous acid.

From results of dialysis and hydrolysis, the Gayoni factor appears to be bound to protein in natural products but is not of protein proportions itself. Maximum growth-promoting activity for *L. gayoni* is supplied by this dissociated form, which is much more stable to hydrolysis than is the complex from which it is derived. These facts alone would seem to distinguish it from the chick vitamins B<sub>10</sub> and B<sub>11</sub> (20, 21) or the *L. casei* yeast factor (7), all of which may be large molecule conjugates of some of the various "folic acids." This yeast factor, which successfully replaces the vitamin M required by the rhesus monkey (22), showed no activity when tested by *L. gayoni*.

Members of the folic acid group which were tested were crystalline vitamin B<sub>6</sub> (8), crystalline liver folic acid and a folic acid concentrate from spinach (6). None of these could replace the Gayoni factor. The latter may thus be regarded as also different from Peterson's Norit eluate factor (23), the *L. casei* liver factor (24), and the chick factor R reported by Hill and coworkers (25) which may be similar to vitamin B<sub>11</sub> (25).

Other factors which differ from the Gayoni factor in one or more respects are: (1) Hill's factor S (25), which is not readily adsorbed on common adsorbents and which may be identical (25) with the kidney residue factor of Mills *et al.* (26); (2) two similar growth factors reported by Woolley (10, 11), termed "strepogenin" and GPF-3, which are not precipitated by lead and are not adsorbed by Norit; (3) the *S. lactis* factor of Smith (27), which is not precipitated by lead or

mercury and is not adsorbed on Darco or fuller's earth; (4) the tomato juice factor of Kuiken *et al.* (28), which withstands 24-hour refluxing with 8 *N* H<sub>2</sub>SO<sub>4</sub>; (5) the diphtheria bacillus factor of Chattaway (29, 30), which fails to adsorb on fuller's earth, is not precipitated by lead, mercury or phosphotungstic acid, and is not destroyed by excess bromine; (6) an *L. casei* growth factor reported by Chattaway (31), which fails to adsorb on fuller's earth at pH 3; (7) the growth factor reported by Ballentine (32) for a gas gangrene *clostridium*, which is not adsorbed on Norit at pH 3, is not precipitated by silver or phosphotungstic acid, and fails to be oxidized with KMnO<sub>4</sub> at pH 7.5; (8) Barton-Wright and coworkers (33) report isolation of four growth factors from liver which are necessary for *L. helveticus* and *S. lactis* R. Of these, only HL 4 (as distinguished from HL 1, 2 and 3) was affected by nitrous acid treatment. HL 4 is, however, a portion of the fraction soluble in chloroform.

The possibility that the Gayoni factor may be a multiple entity must, of course, be considered. However, our experience supports the belief that it is a single substance. The agreement among assay values obtained at different levels is usually good regardless of the potency of the concentrates, and at no time has the activity of combined fractions appreciably exceeded the sum of the activities of the individual fractions. On the contrary, it is frequently observed that the sum of these activities is greater after fractionation, particularly after adsorption on Lloyd's reagent. This has been attributed to the removal or inactivation of a growth inhibitor by the Lloyd's reagent. Final proof of the identity of the Gayoni factor must await its further concentration and isolation in pure form. Studies of this nature are in progress.

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### SUMMARY

A new growth factor is reported which is essential to growth of the bacterium *Lactobacillus gayoni* 8289. A microbiological assay method has been developed, and the factor has been distinguished from 66 organic compounds and growth factor concentrates. Its properties appear to differentiate it from other unidentified growth factors recently reported in the literature. Preliminary classification and characterization studies have been made, and a 40-fold concentration of the factor has been effected. Further concentration studies are in progress.

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# The Excretion of B-Complex Vitamins in the Urine and Feces of Seven Normal Adults

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## INTRODUCTION

Many investigators have reported unallied normal daily *urinary* excretions of some of the B-complex vitamins and their derivatives (1, 2, 3, 4, 5, 6, 7, 8, 9). Some investigators have also reported on the *fecal* output of a few of these B-vitamins (1, 3, 7, 8, 10). Balance studies of young women on a milk diet showed that the total output of biotin was slightly greater than intake, 107%, while that of pantothenic acid almost equalled the intake and that of riboflavin amounted to only 55% of the intake (1). On an unrestricted diet normal subjects excreted in the feces and urine three to six times the amount of biotin ingested in the diet (3).

Since there are, however, very few reports on the coexisting dietary intake and total excretion of the lesser known B-complex vitamins by human subjects, such values for seven healthy young men eating a



normal diet are reported in this paper. This study was undertaken preliminary to an investigation of the effects of a decreased intake of the B-vitamins (24). During a twelve-week period the urinary and fecal excretions of *p*-aminobenzoic acid, biotin, folic acid, pantothenic acid, pyridoxine, thiamine, riboflavin and nicotinic acid have been compared with the dietary intake of these vitamins. No report of similar studies of this scope has been found in the literature.

## EXPERIMENTAL

*Subjects.* The subjects were seven volunteers, 23 to 28 years old, judged to be free from significant organic disease or defects. These men were housed under identical conditions in a hotel, and later a student dormitory. All ate the same diet, which remained unchanged during the entire experimental period. Strict adherence to the regimen was assured by the high character and scientific interest of the subjects, who were carefully chosen on the basis of their past records under similar experimental conditions. (As added precaution, each week's data were carefully scrutinized for signs of any possible deviation in intake of food, excretion of urine and feces, collection of samples and technical error in assay.) A constant body weight was maintained by regulating physical activity. The principal activity was provided by standardized daily tasks, *e.g.*, bicycle riding, treadmill, outdoor walks, etc. The remaining free time was spent by participating in various sports and a schedule of maintenance and assistance work.

*Diet.* All meals were prepared and served under the direct supervision of a trained dietitian. The menu was followed strictly and all portions were carefully weighed. Extra identical meals were prepared one day each week, carefully ground, homogenized, frozen and used for assay. The meat used was bought daily, as was the milk. The fresh vegetables were purchased three times each week at local wholesale dealers. The canned foods were purchased in one lot of the same pack in a quantity sufficient for the entire experiment. It was computed that a total of 3000 to 3300 calories daily was provided, 70 g. of protein, 860 mg. of calcium, 1.25 g. of phosphorus, 15 mg. of iron, 7400 I.U. of Vitamin A and 100 mg. of ascorbic acid. The determined (by analysis) average daily intakes of the B-complex vitamins under consideration in this report are listed in Table II.

*Urinary and Fecal Collection.* Urine was collected each week over a period of four consecutive days. The containers, amber-colored one-gallon jugs, were kept in a refrigerator at all times. At the end of each day the total volume and a one-tenth aliquot were measured. The aliquot of each daily sample was transferred to a sample bottle which was kept in the deep-freeze unit.

Feces were also collected over the same four-day period each week. All feces from the entire period were collected in one container, a tared one-quart jar, which was kept in a deep-freeze unit at all times. Shortly after the end of the four-day period the feces were thawed and homogenized. Daily average excretion was computed and aliquots taken for assay. In two cases fecal collection was continued beyond the four-day period until a minimum of three stools had been collected.

*Methods.* For the determination of thiamine the method of Hennessy and Cerecedo (11), as modified by Friedemann and Kmiecik (12), was used. For riboflavin the method of Ferrebee (13) was used with slight modification. Folic acid was determined microbiologically using the method of Topley and Elvehjem (14). Feces and food samples were prepared for folic acid, pantothenic acid and biotin assays by the procedures outlined by the University of Texas workers (15). The biotin value for food obtained by the enzyme method was checked occasionally by autoclaving a duplicate sample with HCl for  $\frac{1}{2}$  hour, neutralizing and assaying. Pantothenic acid was determined microbiologically by the method of Hoag *et al.* (16). In this method crystalline vitamin solutions were substituted for the yeast and Vitab mixtures. Biotin was determined microbiologically originally by the method of Shull and Peterson (17). Later the method of Topley and Elvehjem for folic acid was used for biotin assays. *p*-Aminobenzoic acid was determined with *Neurospora crassa* by using a method based on that of Thompson *et al.* (18) and that of Stokes *et al.* (19). Pyridoxine was determined by a method based on that of Stokes and co-workers (19). Nicotinic acid was determined microbiologically by the method of Krehl and co-workers (20). N<sup>1</sup>-methylnicotinamide was determined by a modification of Najjar's method (21).

## RESULTS

Daily urinary and fecal excretions of the B-vitamins are shown in Table I. These values represent the average of six to eight weekly determinations (samples collected for four consecutive days each week) made during a twelve-week period. More variation is noted in the fecal output than in the urinary excretion. Individual output varied more from week to week than did the average between individuals in any one week.

About twice as much thiamine and nicotinic acid was found in the feces as in the urine. About one and one-half times as much riboflavin and *p*-aminobenzoic acid was found in the feces as in the urine. Urinary biotin output was less than one-fourth as great as the fecal output. About seventy-five times as much folic acid was excreted in the feces as in the urine. However, the major part of the pantothenic acid and pyridoxine excreted appeared in the urine, only about 40% of the total excretion of these vitamins appearing in the feces.

The combined urinary and fecal output of thiamine, nicotinic acid and pyridoxine was considerably less than the dietary intake. The urinary plus fecal output of riboflavin was only slightly below the dietary intake. The ratio of urinary plus fecal output to dietary intake of biotin was about 3.8 to 1, pantothenic acid about 1.1 to 1, *p*-aminobenzoic acid about 2.3 to 1 and folic acid about 5.5 to 1, as shown in Table II.

TABLE I  
Average\* Daily Urinary and Fecal Excretions

Urine Subject	p-Amino-benzoic acid Low-High-Ave.	Biotin Low-High-Ave.	Folic acid Low-High-Ave.	Pantothenic acid Low-High-Ave.	Pyridoxine Low-High-Ave.	Nicotinic acid Low-High-Ave.	N <sup>5</sup> -methyl-nicotinamide Low-High-Ave.	Thiamine Low-High-Ave.	Riboflavin Low-High-Ave.
1.	73-261-131	23.7-31.5-28.0	2.05-5.05-4.28	2.88-3.48-3.19	0.83-1.12-0.61	0.70-1.40-1.18	2.3-5.4-3.9	224-328-261	470-045-758
2.	85-308-148	27.4-45.5-35.5	2.06-4.39-3.91	2.88-3.90-3.37	0.34-1.01-0.60	0.95-1.37-1.13	2.0-4.3-3.1	252-382-323	498-870-694
3.	89-246-149	21.2-39.8-27.8	2.54-5.80-4.01	2.93-3.18-3.07	0.38-0.96-0.57	1.00-1.37-1.23	1.3-4.8-3.3	128-204-156	388-697-543
4.	67-260-132	19.7-39.8-29.7	2.35-5.60-3.91	2.31-2.96-2.57	0.36-0.99-0.63	0.96-1.42-1.19	2.4-4.8-3.4	184-273-227	437-747-388
5.	60-280-144	22.7-49.3-33.1	2.05-4.80-2.64	3.01-4.13-3.46	0.23-1.16-0.69	0.97-1.30-1.16	2.4-5.6-3.7	178-282-234	464-735-601
6.	89-494-198	23.6-44.4-32.5	2.04-4.99-3.66	2.40-3.19-2.68	0.38-1.17-0.67	1.15-1.55-1.39	2.0-4.1-2.7	203-312-245	475-788-651
7.	70-272-132	28.7-47.3-35.6	3.88-5.90-4.99	2.65-3.30-2.95	0.32-1.26-0.64	0.90-1.40-1.20	2.8-6.1-4.4	110-184-144	593-1120-913
Range of Averages	131-198-148	27.5-35.6-31.7	2.04-4.99-3.99	2.08-3.46-3.04	0.37-0.60-0.63	1.13-1.30-1.21	2.7-4.4-3.5	144-323-227	543-913-678
Feces									
1.	71-336-200	59-171-121	213-350-294	0.69-1.63-1.16	0.17-0.46-0.33	0.84-2.96-2.14	2.2	564-900-755	632-1204-869
2.	177-280-238	70-465-201	189-536-340	0.27-1.10-0.89	0.17-0.73-0.36	1.93-3.78-2.49		449-887-589	649-1589-940
3.	84-345-249	62-210-131	322-522-393	2.72-4.34-3.66	0.22-0.46-0.38	4.23-6.37-5.41		611-700-651	1159-1450-1313
4.	72-365-255	69-225-125	179-342-281	2.13-4.12-2.63	0.26-0.54-0.42	2.89-5.31-4.17		71-208-130	754-1180-972
5.	187-301-239	90-201-125	273-501-356	1.50-3.63-2.36	0.25-0.55-0.38	2.45-6.24-3.99		745-1217-895	775-1480-1117
6.	104-276-183	44-230-116	135-308-222	0.68-2.95-1.39	0.19-0.55-0.24	1.17-3.50-2.24		47-175-109	563-1034-823
7.	158-674-361	51-186-114	123-395-243	1.67-4.11-3.04	0.27-0.50-0.42	3.08-7.96-4.95		450-860-699	825-1445-1172
Range of Averages	183-361-246	114-201-133	222-392-304	0.89-3.66-2.20	0.33-0.42-0.38	2.14-5.41-3.63		109-895-548	823-1313-1029
Excl. No	repre- sented.								

TABLE II  
*Comparison of Daily Vitamin Intake\* and Urinary Plus Fecal Excretion\*\**

Urinary plus Fecal Output Subject	p-Amino- benzoic acid Low-High-Ave.	Biotin Low-High-Ave.	Folic Acid Low-High-Ave.	Pantothenic Acid Low-High-Ave.	Pyridoxine Low-High-Ave.		Nicotinic Acid Low-High-Ave.		Thiamine Low-High-Ave.		Riboflavin Low-High-Ave.
					mg.	mg.	mg.	mg.	mg.	mg.	
1.	150-448-331	87-200-149	217-361-298	3.78-4.76-4.36	0.50-1.43-0.94	1.87-4.20-3.32	1.87-4.20-3.32	0.89-1.18-1.02	1.45-1.87-1.63		
2.	283-598-386	116-493-236	192-549-344	3.44-4.98-4.27	0.62-1.31-0.96	2.44-5.11-3.48	2.44-5.11-3.48	0.79-1.26-0.93	1.37-2.23-1.64		
3.	173-509-398	83-292-159	226-526-397	5.90-7.21-6.72	0.63-1.42-0.95	5.47-7.52-6.64	5.47-7.52-6.64	0.76-0.88-0.81	1.60-2.12-1.88		
4.	139-517-387	92-252-155	183-440-289	4.51-6.92-5.49	0.69-1.36-1.05	4.03-6.73-5.36	4.03-6.73-5.36	0.29-0.48-0.37	1.39-1.83-1.57		
5.	254-477-383	125-299-158	277-503-360	4.80-7.33-5.82	0.48-1.58-1.07	3.42-7.42-5.15	3.42-7.42-5.15	0.98-1.41-1.13	1.51-2.06-1.73		
6.	209-670-365	77-236-136	139-310-226	3.34-5.75-4.07	0.57-1.09-1.03	2.42-3.17-3.62	2.42-3.17-3.62	0.25-0.49-0.36	1.15-1.69-1.48		
7.	158-574-361	86-233-150	127-401-248	4.60-7.41-5.99	0.62-1.73-1.05	4.78-9.29-6.15	4.78-9.29-6.15	0.57-1.04-0.85	1.68-2.47-2.11		
Range of Averages	331-393-373	136-236-163	226-397-310	4.07-6.72-5.25	0.94-1.07-1.01	3.32-6.64-4.82	3.32-6.64-4.82	0.36-1.02-0.78	1.48-2.11-1.72		
Average Daily Dietary Intake	97-220-188	37-54-44	43-36-62	4.10-5.30-4.73	1.32-2.46-1.76	12.4-20.9-15.6	12.4-20.9-15.6	1.24-1.63-1.44	1.74-1.93-1.84		
Per cent Excretion = $\left( \frac{\text{Urinary} + \text{Fecal Excretion}}{\text{Dietary Intake}} \times 100 \right)$											
1.	131-289-209	265-460-367	477-720-539	71-110-93	43-78-57	13-27-31	13-27-31	67-87-80	80-97-88		
2.	163-564-280	281-1040-490	297-847-613	70-116-93	44-86-56	19-27-22	19-27-22	58-93-72	72-112-90		
3.	156-355-233	324-464-369	470-811-643	106-177-144	41-70-48	36-54-43	36-54-43	56-65-60	84-109-96		
4.	153-313-223	250-405-351	425-581-521	92-165-122	45-81-62	32-40-35	32-40-35	22-35-28	75-92-82		
5.	146-310-220	298-757-409	461-812-655	85-175-114	43-81-66	27-42-34	27-42-34	72-84-80	81-100-92		
6.	103-335-226	188-608-334	322-485-380	60-137-87	42-87-57	17-30-22	17-30-22	20-28-23	66-85-79		
7.	121-297-217	210-564-346	198-729-442	87-177-130	40-84-55	34-44-40	34-44-40	46-68-57	96-125-108		
Range of Averages	209-280-230	331-490-378	380-655-542	87-144-112	48-68-57	21-43-31	21-43-31	23-50-57	79-108-91		

\* Each figure represents a random one-day sample.

\*\* Each figure represents a four-day sample.

## DISCUSSION

The results of this study confirm the findings of other workers that the output of biotin in urine plus feces is greater than the dietary intake. In addition, evidence is presented suggesting that in normal young men subsisting on a normal diet, intestinal bacteria synthesize large quantities of pantothenic acid, *p*-aminobenzoic acid, biotin and folic acid. Possibly nicotinic acid is also synthesized in appreciable quantities by intestinal bacteria. *N*<sup>1</sup>-methylnicotinamide, which was not included in our values for total excretion, appears in urine to the extent of thrice the quantity of niacin. Other workers (5) report over 94% of the total nicotinic acid and metabolites excreted in the urine is in the form of *N*<sup>1</sup>-methylnicotinamide. If the latter substance appears in feces in quantities 2 or 3 times as great as the niacin value, total urinary and fecal excretion would equal or slightly exceed the intake.

In addition to pantothenic acid, *p*-aminobenzoic acid, biotin and folic acid, the bacteria of the intestine probably synthesize much greater quantities of pyridoxine than our data indicate. The organism we used, *Neurospora sitophila* 299, measured pyridoxine, pyridoxamine and pyridoxal (22). Another metabolite of pyridoxine, 4-pyridoxic acid, has been reported to be excreted in much greater quantities in the urine than the aforementioned three substances (9). It is quite possible feces may contain additional quantities of 4-pyridoxic acid. Thus, the figures we present do not represent the entire excretion of nicotinic acid and pyridoxine and their metabolites. The true values are probably much greater than those reported here. This is perhaps true of the other vitamins where the metabolic products are not yet known or determinable.

Dermal excretion of thiamine, riboflavin, biotin, pantothenic acid, *p*-aminobenzoic acid, pyridoxine and nicotinic acid is reported to be practically negligible as compared with the urinary excretion (6, 23). However, some folic acid is reported to be excreted in the sweat (6), but since the fecal excretion is large, this amount would be relatively negligible under the conditions of this experiment.

From these data it is apparent that on a normal diet a normal individual excretes in the urine and feces far more *p*-aminobenzoic acid, biotin, folic acid and pantothenic acid, and possibly more pyridoxine

and niacin, plus their metabolites, than is ingested in the diet. Possibly the synthesis of these vitamins in the intestine by bacteria accounts for these results. The question of whether the large quantity of B-vitamins eliminated in the feces is excreted because it is not needed or because it is not available to the body is of much interest. In these studies it was found that between 60–100% of each of the B-vitamins in feces was extractable with water.

There was no parallel in any of the individuals concerned between dietary intake and urinary plus fecal excretion. There appeared to be a familial resemblance between the urinary and fecal excretion of thiamine in subjects 4 and 6, who are brothers. In these instances, the total excretion (urinary plus fecal) as expressed in terms of percentage of the intake, was similarly low for each subject. Similar findings were obtained in these individuals for riboflavin. (See Table II.)

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### SUMMARY

1. Daily urinary and fecal excretion of *p*-aminobenzoic acid, biotin, folic acid, pantothenic acid, pyridoxine, thiamine, riboflavin and nicotinic acid of seven normal young men were determined.

2. Daily dietary intake of these vitamins was determined and compared with the total urinary and fecal output. Daily fecal excretion was larger than urinary excretion in all cases except pantothenic acid and pyridoxine.

3. The total daily urinary and fecal output of *p*-aminobenzoic acid, biotin, folic acid and pantothenic acid exceeded dietary intake. Dietary intake of thiamine, riboflavin, pyridoxine and nicotinic acid exceeded the combined urinary and fecal output. However, the methods employed do not measure all the metabolites of pyridoxine and nicotinic acid.

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# Quantity and Quality of Amylase Produced by Various Bacterial Isolates<sup>1</sup>

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## INTRODUCTION

The results of screening some thousand or more bacterial isolates for amylase production has been reported by Peltier and Beckord (1). They found 265 isolates capable of hydrolyzing starch in a starch-agar medium. Of these, 71 produced sufficient amylase when grown in a wheat bran-peptone-phosphate liquid medium to be evaluated by the methods they employed. Preliminary characterization of these isolates indicated that they all appear to fall into the *Bacillus subtilis* group.

One isolate from this group has been described in previous communications (2, 3, 4). The amylase produced by isolate 23 was found to differ from the amylase of commerce in having a much higher starch saccharifying action per unit of dextrinizing activity. Further, it was strongly inhibited by an amylase inhibitor present in wheat, whereas the commercial bacterial amylase was not affected. The relationship between the starch-dextrinizing and starch-saccharifying activities of amylase systems appears to be a valuable means of differentiation, particularly when the saccharification procedure is sufficiently prolonged to permit adequate expression of the enzyme properties (5).

Amylase production by bacterial cultures other than those from the *B. subtilis* group has been described. Tilden and Hudson (6) adequately discussed the literature

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relative to the production of amylase by *Bacillus macerans* and *Bacillus polymyxa* and presented their own findings on the preparation and properties of these amylases. Relative production of amylase by several species of the genus *Bacillus* and by two of the genus *Sarcina* was investigated by Janke and Schaefer (7). The amylase produced by *Clostridium acetobutylicum* has been described in some detail (8). Some of the properties of amylases from organisms classified as *Phytomonas* and *Actinomyces* are given by Bois and Savary (9).

The results reported in the literature are somewhat difficult to interpret with respect to relative quantities of amylase produced by various organisms under comparable cultural conditions and with respect to the relative starch degrading properties of the various enzyme systems. It is the purpose of the present communication, and of those in course of preparation, to supply additional information dealing with some of the bacterial amylase systems. Through such studies it is hoped that the properties of the amylases can be more clearly visualized and possible industrial applications investigated.

From the 71 amylase-producing isolates of the *B. subtilis* group (1) 43 were selected for additional investigation. For comparison, 7 cultures of *B. polymyxa* and 3 of *B. macerans* were obtained.<sup>4</sup> These 53 organisms were cultured comparably in a liquid medium found in former studies to be "adequate" for bacterial growth and amylase production. The quantity of amylase produced was evaluated and some of the properties of the amylase systems compared. The present report deals with a few of the general characteristics of the amylases; a more detailed comparison of the amylases from typical organisms is being prepared for publication.

## EXPERIMENTAL

### Methods

*Media and Culture Conditions.* The liquid medium for growth of the *B. subtilis* isolates was prepared in the following manner: a water extract of wheat bran was obtained by autoclaving a bran-water mixture (5 parts of wheat bran to 100 parts of distilled water) at 15 lbs. pressure for 20 minutes, followed by straining through cheesecloth. In each liter of the strained liquid 10 g. Bacto-peptone (Difco), 0.7 g.

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<sup>4</sup> Grateful acknowledgment is hereby made to Mr. N. R. Smith of the Bureau of Plant Industry, Soils and Agricultural Engineering, U. S. Department of Agriculture, who kindly supplied most of the *B. polymyxa* and *B. macerans* cultures, and to the Northern Regional Research Laboratory of the U. S. Department of Agriculture for two of the cultures.

$K_2HPO_4 \cdot 3H_2O$  and 0.3 g.  $KH_2PO_4$  were dissolved. Aliquots (200 ml.) were dispensed into appropriate glass culture vessels and autoclaved again for 20 min. at 15 lbs. pressure. The depth of liquid in the culture vessels was approximately 3 cm. To 200 ml. volume of culture liquid were added the washings (in 5 ml. of water) from a "bran-agar" slant of the bacteria previously incubated at 35°C. for 24 hours. The medium for the growth of the inocula was prepared by adding 1.5% agar to a liquid identical with the above liquid medium (bran extract-peptone-phosphate).

The liquid medium for growth of the *B. polymyxa* and *B. macerans* cultures was identical to that described above with the exception that 20 g. of  $CaCO_3$  were added per liter of liquid to neutralize the acids produced by these organisms.

Growth of all the organisms was for 4 days at 35°C. (Numerous experiments demonstrated that approximately maximum amylase activity was attained in this period.) The liquid cultures were then centrifuged and the centrifugates stored under refrigeration until the following day when dextrinizing activity determinations were made. Starch saccharification data were obtained during the next few days.

*Evaluation of Starch-Dextrinizing Activity.* A property common to the bacterial amylases appears to be their ability to hydrolyze gelatinized starch to low-molecular dextrans which give essentially no color with iodine. Routine activity evaluations were most conveniently made by use of a starch dextrinization procedure, a modification of the well-known Wohlgemuth (10) method. The substrate was a 20 ml. aliquot of 1% boiled soluble starch buffered with phosphates (3.0 g.  $KH_2PO_4$  and 0.6 g.  $K_2HPO_4 \cdot 3H_2O$  per liter) to a pH value of 6.0. The time, in minutes, required by an appropriate aliquot of the liquid culture to convert the substrate at 30°C. to a point where the "red-brown" color is given with iodine (11) was determined for all of the cultures except those of *B. macerans*. At no stage of the dextrinization did the products from the action of *B. macerans* amylase give the typical color with iodine. Accordingly, it was necessary to evaluate the amylase activities of these organisms by selecting another "end point"; for convenience, the one selected was the point at which 1 ml. of the reaction mixture just failed to impart a color to the standard iodine test solution (11). For malt  $\alpha$ -amylase it requires almost 2.5 times as long to carry the hydrolysis to this point as it does to the stage giving the red-brown color.

The aliquots of the cultures used for activity determinations varied from 0.5 ml. to 10 ml., depending on the previously established ability to produce amylase. Since the total reaction volume was a constant 30 ml., it was necessary to adjust the volume when less than a 10 ml. enzyme aliquot was employed; this was done with distilled water. The dextrinization time being inversely proportional to the quantity of amylase added, it was possible, as well as convenient, to record the activities of all the organisms as the time in minutes required for a 10 ml. aliquot of the unconcentrated liquid to dextrinize the starch.

*Response of the Amylases to the Inhibitor of Wheat.* It has been demonstrated (4) that wheat contains a protein-like substance which exercises a selective inhibiting action on the amylases. Some of the bacterial amylases were inhibited whereas others were not.

Dextrinizing activities were determined for the 53 cultures both with and without the inhibitor. The procedure for evaluating response

to the inhibitor was to add a solution of the inhibitor to the enzyme incubate at 30°C. for exactly 5 minutes, and then add the 20 ml. aliquot of starch suspension at 30°C. Dextrinization time was determined as customary. An alcohol-precipitated preparation of the inhibitor (4) was used. For the sensitive amylases only 1 mg. of the material was required to give inhibition. Those preparations which showed no loss in activity in the presence of 1 mg. of inhibitor were further tested with 3 mg. portions.

For a constant amount of inhibitor a greater decrease in amylase activity results at lower levels of activity. Accordingly, it would be meaningless to record the response in terms of *per cent* loss of activity with amylases of varying activity. For all cultures the amylase response to the inhibitor is given as positive or negative. In addition a number of the inhibitor-sensitive amylases were adjusted to equal dextrinization times and then tested for inhibition. These data were then calculated as *per cent* loss of activity; *i.e.*, the difference between the uninhibited and inhibited dextrinizing activities in reciprocal minutes related to the uninhibited activity (1/min.) in terms of *per cent*.

*Evaluation of Starch Saccharifying Activity.* It is well known that the hydrolytic products from amylase action on starch may be variable. In addition to sugars, both maltose and glucose, a variety of dextrans result. Such dextrans all have reducing actions on the customary reagents used to evaluate the sugar content of a reaction mixture. Thus the reducing value, calculated as glucose or maltose, may not have a close relationship to the actual content of fermentable sugars. Routine testing of the starch-saccharifying activities of the bacterial amylases was based on measurement of the production of sugars actually fermentable by yeast under conditions relatively optimum for fermentation. In previously reported studies (2, 3) the yeast was "activated" or adequately nourished by the use of dried yeast activator. For further work it was considered desirable to refine the procedure. The combined starch hydrolysis-yeast fermentation procedure finally devised is the result of considerable experimental work and embodies modifications designed to insure adequate fermentation of the sugars produced by amylase action on starch with a minimum of dependence on either the type of sugar produced or the variability in yeast nutrients found in the enzyme aliquots employed.

As in previous investigations (2, 3), the "pressure-meter" (12) was employed to determine  $\text{CO}_2$  production by the yeast fermentation. To each pressure-meter cup was added 15 ml. of a solution containing 0.6 g. boiled soluble starch, 0.05 g. asparagin, 0.25 g.  $(\text{NH}_4)_2\text{SO}_4$ , 0.016 g.  $\text{NaCl}$ , 0.04 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04 g.  $\text{KH}_2\text{PO}_4$ , 0.011 g.  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  and 0.03 g. dextrose. The amylase aliquot desired plus sufficient distilled water, if necessary, to give an addition at this point of 10 ml. was added to the above. The contents of the cup were incubated at  $30^\circ\text{C}$ . for 60 min. and then 5 ml. of a suspension containing 0.5 g. baker's compressed yeast, 0.00008 g. thiamine and 0.00008 g. pyridoxine were added. The pressure-meter top was affixed and incubation continued at  $30^\circ\text{C}$ . Pressure was equalized at the end of 5 min. to eliminate the variable pressure produced by introducing the closed system into the water bath.

The fermentation mixture differs from that previously employed (2) by the use of a 30 ml. reaction volume and by the substitution of yeast nutrients and dextrose for the "dried yeast activator." The larger volume permits greater latitude in operation without interfering with the course of fermentation. The yeast nutrients are essentially those of the "complete" nutrient of Atkin, Schultz and Frey (13). The citrate used by them for preliminary buffering was replaced with phosphates. In accordance with their finding that the effect of nicotinic acid was inappreciable, it too was omitted. Equally as good fermentation was possible by substituting 0.2 g. of asparagin for the combination of asparagin and  $(\text{NH}_4)_2\text{SO}_4$  employed. However, when determining the activities of many hundreds of samples the combination was preferable in order to economize on the relatively expensive asparagin.

At least some of the amylase preparations produce maltose as the principal fermentable product of starch hydrolysis. This sugar is only slowly fermented by baker's yeast in the absence of either dextrose or an enzyme, maltase, which can convert the maltose to glucose. Schultz and Atkin (14) showed that part of the effect of dried yeast on maltose fermentation is the result of maltase action and demonstrated the accelerating action of small increments of dextrose. Accordingly, when devising the conditions for rapid fermentation of starch hydrolysis products, it was necessary to investigate the influence of small increments of dextrose on maltose fermentation. It was found that, when added to a mixture of 500 mg. of maltose and the yeast nutrients known to be essential, the first few mg. of dextrose had a very marked accelerating action on fermentation. This acceleration diminished with increasing increments of dextrose. The addition of dextrose used as routine procedure, 30 mg. per 600 mg. of starch, proved to be above the levels causing marked acceleration, and fermentation in its presence gave results closely comparable to those previously obtained when the dried yeast activator of Blish and Sandstedt (15) was employed.

In presenting the results of the combined starch-hydrolysis yeast-fermentation studies it is most informative to give the complete

fermentation curves (2). However, with routine studies the record of the gas pressure, in mm. of Hg, produced during the first several hours, and then again following some 20 hours fermentation, serves to illustrate the broad differences obtained. The blank values attributable to the CO<sub>2</sub> produced by fermentation of the sugars present in the enzyme addition and the dextrose in the nutrient are subtracted in each case. To facilitate comparison of the starch-degrading properties of the various amylases the customary procedure, when practicable, was to adjust the aliquots used for saccharification to give equal dextrinization times. Then differences in fermentation values reflected inherent differences in the enzyme systems.

## RESULTS

*Amylase Activity.* The amylase activities developed by the growth of the 43 isolates belonging to the *B. subtilis* group are listed in Table I. The numbers listed for the various isolates are those assigned by the Department of Bacteriology of the University of Nebraska as the isolations were made. The prefix "S" indicates isolates obtained by Floyd

TABLE I

*Starch Dextrinizing Activity and Response to an Inhibitor of Amylases Produced by Isolates of the Bacillus subtilis Group*

Isolate	Source	Dextrinization time in min. (10 ml.)	Response to inhibitor	Isolate	Source	Dextrinization time in min. (10 ml.)	Response to inhibitor
S-1	Air	23.0	+	W-43	Peanut meal	53	+
S-4	Air	24.0	+	W-92	Peanut meal	7.5	+
S-17	Air	62	+	W-101	Peanut meal	22.0	+
S-18	Air	15.0	+	W-78	Starch	64	+
S-19	Air	44	+	W-96	Starch	6.0	+
S-20	Air	18.0	+	W-97	Starch	19.0	+
S-24	Air	8.5	+	W-98	Starch	12.5	+
S-30	Air	20.0	+	W-85	Potato flour	18.5	+
S-33	Air	13.3	+	W-89	Rice flour	7.5	+
S-34	Air	34	+	W-20	Soil	26.0	+
S-36	Air	27.0	+	P-80	Fish meal	35	+
S-47	Air	17.5	+	P-82	Manure	8.0	+
S-50	Air	37	+				
S-52	Air	12.5	+	W-106	Ropy bread A	0.65	—
P-65	Soybean meal	4.3	+	W-107	Ropy bread A	0.28	—
P-67	Soybean meal	8.5	+	RB-2	Ropy bread B	0.63	—
W-40	Soybean meal	39	+	RB-8	Ropy bread B	0.65	—
W-23	Corn meal mash	17.5	+	RB-10	Ropy bread C	1.05	—
				RB-14	Ropy bread C	0.80	—
W-65	Stillage (contaminants)	20.5	+	RB-16	Ropy bread D	1.90	—
W-68	Stillage (contaminants)	31	+	RB-17	Ropy bread D	29.0	—
W-69	Stillage (contaminants)	26.0	+				
W-72	Stillage (contaminants)	17.5	+				
W-103	Stillage (contaminants)	26.0	+				

Schroeder, "W" by Lillian Wind, "P" by Mildred Penner, and "R B", rony bread isolates, by Dr. Geo. L. Peltier. The sources of the isolates are also given, primarily for the purpose of illustrating the sharp differentiation between those obtained from rony bread and those from other materials.

The data of Table I indicate that a separation of the *B. subtilis* isolates into 2 groups can be made on the basis of response to the amylase inhibitor from wheat. The amylases produced by the rony bread isolates are not inhibited, whereas those from all other materials are. Further, it is notable that, with one exception, the rony bread isolates had a much greater capacity for amylase production than the others. The best rony bread isolate, W-107, produced 15 times more amylase than the best of the "inhibited type," P-65. The range in activity found within the inhibited type is very great—from isolates such as P-65, W-96, W-92, W-89, P-82, P-67, and S-24 represented by dextrinization times of less than 10 min., to those of the *B. subtilis* group not reported in the study which produced essentially no amylase when cultured under the same conditions. In contrast, no isolate from any of the 4 different rony bread samples ever failed to give appreciable amylase activity when cultured under favorable conditions; of the 24 isolates made from rony bread (8 of these are reported in Table I) only one, RB-17, gave other than a high amylase activity.

Cultures of *B. polymyxa* and *B. macerans* resulted in amylase production similar in quantity to that produced by the "inhibited type" of *B. subtilis*. The numbers and sources of the cultures together with the amylase activities found and the responses to wheat inhibitor are listed in Table II. One culture, N. R. Smith 354, can be considered as a relatively efficient amylase producer since the activity found in liquid culture was comparable to the highest levels produced by the inhibited type of *B. subtilis*. Tilden and Hudson (6) likewise found this culture of *B. polymyxa* to be superior in its amylase-producing power. The growth period in the present experiment was only 4 days as compared to the 3 weeks used by Tilden and Hudson (6).

It has been noted above that the dextrins produced by *B. macerans* hydrolysis of starch at no stage give the standard red-brown color with iodine. Instead of the color changing from blue, through violet, red-violet, clear red-brown, lighter brown, and then to colorless, a brown violet changing to cloudy brown coincides with the stage of hydrolysis associated with attainment of the customary "end-point." These

colors have been described as typical of the formation of Schardinger dextrins (6). When the reaction was allowed to proceed at 30°C. the depth of brown coloration given with the standard iodine reagent gradually became less until essentially no color was imparted. The dextrinization times given in Table II are those required for 10 ml.

TABLE II

*Starch Dextrinizing Activity and Response to an Inhibitor of Amylases Produced by Cultures of B. polymyxa and B. macerans*

Cultures	Type	Source	Dextrinization time-min (10 ml)	Response to inhibitor
354	<i>B. polymyxa</i>	N. R. Smith	5.5	—
251	<i>B. polymyxa</i>	N. R. Smith	13.5	—
813	<i>B. polymyxa</i>	N. R. Smith	16.0	—
280	<i>B. polymyxa</i>	N. R. Smith	22.0	—
279	<i>B. polymyxa</i>	N. R. Smith	22.0	—
391	<i>B. polymyxa</i>	N. R. Smith	59	—
510	<i>B. polymyxa</i>	N. R. R. L.†	45	—
B-394	<i>B. macerans</i>	N. R. R. L.†	50*	—
277	<i>B. macerans</i>	N. R. Smith	36*	—
278	<i>B. macerans</i>	N. R. Smith	69†	—

† Northern Regional Research Laboratory.

\* Time to colorless with iodine.

aliquots of the liquid cultures to cause this change. Obviously the results are not directly comparable with those found for the other amylases. If it is assumed that the time taken to reach a stage of hydrolysis somewhat comparable to that measured for the others is from one-half to one-third that indicated, it becomes apparent that amylase production by the *B. macerans* cultures is similar in quantity to those found for many of the *B. polymyxa* cultures and *B. subtilis* isolates. The N. R. Smith culture 277 proved to be a superior strain in accordance with the findings of Tilden and Hudson (6). Also in accordance with their results, the original Schardinger culture, in this case NRR L B-394, was of low potency.

The amylases of the *B. polymyxa* and *B. macerans* cultures were not inhibited by the wheat inhibitor. In this respect they were similar to amylases from the rosy bread isolates of *B. subtilis*.

*Relative Response to Inhibitor.* The culture liquids on which 12 representative isolates of the "inhibited type" of *B. subtilis* grew for 4 days were selected. Aliquots of these were taken in such a manner that each gave a dextrinization time of 20 min. on 1% starch. Each aliquot was incubated with 1 mg. of precipitated inhibitor for 5 min. and then 20 ml. of starch at 30°C. added. The dextrinization times found for the inhibited reactions and the *per cent* reductions of amylase activity caused by the presence of the inhibitor are given in Table III.

TABLE III  
*Relative Response of a Group of Inhibitor-Sensitive Bacterial Amylases to the Inhibitor from Wheat*

Isolate	Dextrinization times		Reduction of amylase activity
	no inhibitor	inhibitor added	
	min.	min.	per cent
W-85	20	100	80
W-89	20	128	84
W-92	20	116	83
W-96	20	119	83
W-98	20	118	83
W-23	20	79	75
P-82	20	90	78
P-67	20	119	83
P-65	20	100	80
Y-33	20	119	83
Y-21	20	130	85
Y-18	20	94	79

It is apparent from Table III that the amylases from all 12 isolates reacted in a similar fashion to the inhibiting substance. Certain differences in response were evident; *e.g.*, the amylase of isolate W-23 was reduced in activity by 75%, that of isolate S-24 by 85%. Though these differences might have been somewhat greater if a lower quantity of inhibitor had been used the results do indicate a rather high degree of uniformity in response and, therefore, a degree of uniformity among the bacterial isolates producing the inhibitor-sensitive amylases.

*Starch Saccharification.* In the conversion of starch to fermentable sugars, based either on the first stage of the reaction or on total conversion, the amylases of all 35 isolates of the inhibited type of *B.*



*subtilis* described in Table I reacted very similarly. That is, when they were adjusted to equal dextrinizing activities they had similar starch saccharification reactions. The same applied to the amylases from the group of isolates obtained from rony bread; they differed widely in properties from the inhibitor-sensitive type but showed only minor variations amongst themselves. Accordingly, saccharification data are given for only several representative members of each of these two groups and the results compared with those obtained for the *B. polymyxa* and *B. macerans* amylases. In addition, fermentation data are given for a sample of commercial type of bacterial amylase (Wallerstein), for a barley malt extract (Fleischmann malt) and for an extract of mold bran (Wallerstein). These represent three of the commercially available amylase systems. The bacterial amylase is produced from a selected isolate of *B. subtilis* (or *B. mesentericus*) on liquid medium, the mold bran by the growth of a selected strain of *Aspergillus oryzae* on cooked wheat bran, and the malt by germination of barley under conditions favorable for the development of the  $\alpha$ - and  $\beta$ -amylase activities. The results are shown in Table IV.

For the comparative fermentation studies recorded in Table IV the culture aliquots employed were adjusted in an attempt to have all the amylases equal in one type of starch degrading activity, dextrinization. With some it was impossible to do this because of limitations in the total volume of liquid permitted by the method. For comparison with these samples of low activity an additional aliquot of W-20 amylase was used. Further, to demonstrate the effect of increasing the concentrations of the "low-saccharifying" amylases, aliquots representing much greater quantities of two types were employed. With isolate W-107 this could be done by using a larger portion of the culture liquid. To obtain a more active preparation of the *B. macerans* type it was necessary to precipitate one of them, B-394, and redissolve the precipitate in a smaller volume of water.

A more detailed comparison of typical amylases from the groups of bacterial cultures will be presented in a later paper. However, the data of Table IV are sufficient to indicate a classification into 4 general groups on the basis of the relationship of starch-saccharifying to starch-dextrinizing activities: (1) an amylase system that causes a rapid conversion of starch to fermentable sugar with fairly high levels of conversion—*B. polymyxa*; (2) a system that is intermediate in the rate of sugar production relative to dextrinizing activity and yields high

TABLE IV

*Conversion of Starch to Fermentable Sugars by Bacterial Amylases*

Isolate	Dextrinization time of aliquot used	CO <sub>2</sub> production after indicated intervals			
		3 hrs	5 hrs	20 hrs	24 hrs
	min.	mm. Hg	mm. Hg	mm. Hg	mm. Hg
<i>B. subtilis</i> (inhibited type)					
S-24	29	59	150	375	379
P-65	29	61	149	369	370
P-82	29	67	157	393	395
W-96	29	48	137	377	383
W-20	29	51	145	397	404
W-20	50	27	100	366	375
<i>B. subtilis</i> (non-inhibited type)					
RB-2	29	2	14	54	56
RB-16	29	2	12	46	47
RB-17	29	1	5	62	72
W-107	29	1	4	45	50
W-107	1.2	57	89	120	121
<i>B. polymyxa</i>					
354	29	226	306	348	349
251	30	264	327	360	375
813	31	239	316	354	362
280	30	219	314	367	369
279	30	234	320	361	363
301	60	202	294	360	364
510	45	114	260	339	340
<i>B. macerans</i>					
277	36*	0	0	24	32
278	70*	0	0	94	98
B-394	50*	0	3	56	61
B-394	30*	0	15	121	125
B-394	5†	135	204	316	323
"Commercial" amylases					
Mold bran†	29	10	49	334	349
Bacterial amylases	29	1	5	50	55
Barley malt	29	227	303	337	342

\* Time to colorless with iodine.

† From the Wallerstein Laboratories.

levels of overall conversion—*B. subtilis* of the “inhibited type”; (3) one that has a very low production of fermentable sugars relative to dextrinizing activity and, even when added at a high enzyme level, does not give extensive conversion—*B. subtilis* of the “non-inhibited” or “ropy bread type”; and (4) a system that produces essentially no fermentable sugars during the early stages of reaction when normal enzyme levels are used, but “does convert starch to sugar if sufficient time is permitted and with considerable efficiency if high enzyme levels are employed—*B. macerans*.

It is obvious from Table IV that this method of characterization of the amylases reveals certain similarities and dissimilarities between the experimental bacterial types and those available commercially. The inhibitor-sensitive amylase produced by *B. subtilis* has somewhat similar starch-degrading properties to the enzyme system produced by *Aspergillus oryzae*. They differ markedly, however, in response to the wheat inhibitor—the fungal amylase is not inhibited. The *B. subtilis* isolates from ropy bread appear to be similar in some respects to the industrial strains used for production of the bacterial amylase of commerce. The amylase from either one has a low ratio of starch saccharifying to dextrinizing properties. Neither one is sensitive to the inhibiting substance in wheat. In addition, most of the ropy bread isolates appear to be potent amylase producers.

The amylase system produced by *B. polymyxa* has a remarkable similarity in starch degrading action to that of barley malt. At a given level of starch dextrinizing activity both have the facility, not only for achieving fairly high levels of starch saccharification, but for causing a rapid conversion of starch to fermentable sugars in the early stages of the reaction.

It must be kept in mind that the method of amylase evaluation employed, combined saccharification and fermentation at comparable dextrinizing activities, is only one procedure for differential evaluation. Additional data (to be published) will expand these relationships. Accordingly, the data of Table IV cannot be used for making fine distinctions between, for example, the starch saccharification efficiencies of the amylase systems of mold bran, barley malt, *B. polymyxa*, and the inhibited or saccharifying type of *B. subtilis*. Rather, their prime value is in the classification of the various enzyme systems into broad groups. Fine distinctions can be based only on a more complete picture of the properties.

## DISCUSSION

Application of amylase evaluating procedures permitting full expression of the properties of the enzyme system being considered may lead to modifications of the prevailing concepts. This is well illustrated by the results found for the amylase system of *B. macerans*. In addition to the recognized conversion of starch to Schardinger dextrans, it now appears that the same enzyme system, or other components of it, causes hydrolysis of these dextrans to fermentable sugar. This additional hydrolytic action may be missed when relatively low concentrations of enzyme are operative over short periods of time, but becomes very pronounced either with extended reaction times or when greater concentrations of the enzyme are introduced.

As inferred previously (2), the term "bacterial amylase" is a misnomer. The studies made so far indicate that, by appropriate selection and culturing of isolates from a bacterial species, a wide variety of amylase systems can be obtained. The system may have the starch-degrading properties characteristic of a true  $\alpha$ -amylase, the properties may approach those of the mixture of amylases present in barley malt, they may lie between these extremes, or they may be unique. There is every reason to believe that amylases differing from those described could result from additional investigation, thus further expanding the types of starch-degrading enzyme systems available both for fundamental studies and for industrial application.

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## SUMMARY

The quantities of amylase produced by 43 bacterial isolates of the *B. subtilis* group, 7 cultures of *B. polymyxa*, and 3 cultures of *B. macerans* have been determined. They were differentiated qualitatively on the basis of the relationship between two types of starch-degrading action—dextrinization and saccharification. A separation is indicated into four general groups with properties as follows:

1. *Bacillus subtilis* (saccharifying type)—These are the common aerobic spore-forming rods usually isolated from plant material. Amylase production varies in quantity from essentially none to rela-

tively high levels. Little sugar production results during starch dextrinization but post-dextrinization saccharification is pronounced and high levels of conversion of starch to fermentable sugars may result. The amylase is sensitive to a wheat inhibitor and is the only one of the bacteria investigated that is retarded in action by the presence of this substance.

2. *Bacillus subtilis* (non-saccharifying or  $\alpha$ -amylase type)—These organisms may be isolated from samples of rye bread. Very high quantities of a characteristic  $\alpha$ -amylase usually result from culturing on an appropriate medium. In all the characteristics investigated the amylase appears to be closely related to the commercial type of bacterial amylase.

3. *Bacillus polymyxa*—These organisms produce an amylase or an amylase system with starch-degrading properties similar to those of a barley malt extract. Production of fermentable sugar is high both in the dextrinization and post-dextrinization stages. Conversion levels comparable to those given by malt are attained.

4. *Bacillus macerans*—A unique type of amylase system is produced by cultures of this organism. Following the initial conversion of starch to the non-reducing, non-fermentable products that have been called "Schardinger dextrins" a progressive production of fermentable sugar takes place. With sufficient time or with high enzyme levels the saccharification proceeds to relatively high levels of starch conversion.

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# Essential Amino Acid Content of Casein and Fresh and Processed Cow's Milk as Determined Microbiologically on Hydrolyzates

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## INTRODUCTION

The amino acid content of casein has probably been investigated more often than that of any other protein. Casein is easily prepared and, although it is probably a mixed rather than a single protein, samples prepared by different investigators and by different procedures are remarkably similar in chemical and physical properties. Since casein constitutes such a large part of the total proteins of cow's milk, a knowledge of its amino acid content provides a good basis for evaluating the amino acid content of milk itself, although such an evaluation obviously neglects the amino acid content of the other milk proteins which may have an abnormally high or low content of a particular amino acid. These facts have contributed to the chemist's interest in casein. The amino acids of lactalbumin have also been investigated and values for the amino acid content of mixed milk proteins are available. These data have been amply reviewed by Block and Bolling (1) and by Williamson (2). Complete amino acid data on  $\beta$ -lactoglobulin have been published by Brand, Saidel, Goldwater, Kassel and Ryan (3).

Because of the widespread use of both fresh and processed milk in human nutrition, particularly in the diet of infants, children and invalids, and because dried milk and milk by-products are useful protein supplements in animal nutrition, further work, which would either confirm or add to present knowledge of the amino acid content of milk proteins, would be of value to the nutritionist.

The development of the microbiological methods of assay for the determination of amino acids has opened new opportunities for fur-

ther investigation of the amino acid content of milk proteins since these methods differ in principle of measurement from the chemical methods and may be applied to hydrolyzates of food-stuffs without separation of the protein. Because of their comparative simplicity, the microbiological methods are already finding widespread use for the determination of the essential amino acid content of foodstuffs.

The present investigation was undertaken for the purposes of obtaining:

1. Microbiological data for comparison with the older chemical data on the amino acid content of milk proteins,
2. Microbiological data which would be comparable to data on other foodstuffs obtained by similar methods,
3. Data on both fresh and processed milk.

#### ORGANISMS

*Lactobacillus arabinosus* 17-5 was used as the test organism for isoleucine, valine and tryptophane; *Leuconostoc mesenteroides* P-60 was used for histidine, lysine, tyrosine, phenylalanine and methionine, and *Streptococcus faecalis* R was used for arginine, leucine and threonine. All assays were conducted titrimetrically. *S. faecalis* was the only organism found suitable for the threonine assay under the conditions tested. The same organism was used for leucine and arginine solely as a matter of convenience, since suitable hydrolyzates, reagents, etc. had been prepared for the threonine assay. Hier, Graham, Freides and Klein (4) have reported the successful use of *L. arabinosus* for threonine analyses with a different medium than we used in our tests. Stokes and Gunness (5) have shown that *L. arabinosus* can synthesize threonine in the presence of pyridoxal or pyridoxamine. Under our conditions *L. arabinosus* required threonine, satisfactory standard curves were obtained and the agreement between assay levels was good. However, the results secured on hydrolyzates of casein and milk were almost exactly double the values found by chemical methods (1, 2) and those found later by the use of *S. faecalis*. Under our conditions *Leuconostoc mesenteroides* also required threonine, as has been shown by Dunn, Shankman, Camien, Frankl and Rockland (6), but the standard curves were irregular, the agreement between different assay levels was poor and the estimated results on milk and casein were even higher than those secured with *L. arabinosus*. For these investigations the hydrolyzates referred to below, prepared by refluxing with hydrochloric acid, and the media referred to below were used without major variation.

In our work with *L. arabinosus* threonine assays no investigation was made of possible pyridoxamine or pyridoxal interference and whether our results might be explained on the basis of such interference has not been excluded or confirmed. It is apparent, however, from our own investigations and those of Stokes and Gunness (5) that care must be exercised in choosing suitable conditions for the threonine assay with *L. arabinosus*. Recently, Greenhut, Schweigart and Elvehjem (7) have also suggested that *S. faecalis* is better suited for threonine assays than *L. arabinosus*.

Snell (8) has reviewed the amino acid requirements of the various lactic acid bacteria and lists threonine as accessory for *L. arabinosus* and essential for *Leuconostoc mesenteroides*.

#### METHODS OF HYDROLYSIS USED

The hydrolyzates for the isoleucine, valine, histidine, lysine, phenylalanine and methionine estimations were prepared by gently refluxing a 1 g. sample of either casein or milk solids with 10 ml. of 20% hydrochloric acid for six hours. The hydrochloric acid was removed by evaporation to dryness in the presence of carbon dioxide. The latter step required about 4 hours, thus the total time in contact with the strong acid was about 10 hours.

The hydrolyzates for arginine, leucine and threonine were prepared as outlined by Stokes, Gunness, Dwyer and Caswell (9). One g. of dried material was autoclaved with 10 ml. of 10% hydrochloric acid for 10 hours at 15 pounds pressure.

A comparison was made of the reflux and autoclave methods using four different samples of casein. The two methods gave, respectively, the following average values in *per cent* of the protein; threonine 4.2 and 4.2, leucine 9.9 and 9.7 and arginine 3.6 and 3.5. Determinations of other amino acids were not made, nor were the two methods compared using milk, as both procedures for hydrolysis appeared to be satisfactory.

Tryptophan and tyrosine were determined on a barium hydroxide hydrolysate. The method of hydrolysis used by Greene and Black (10) was followed. Tryptophan results obtained by the assay were multiplied by two to correct for the racemization which occurs on hydrolysis. The use of alkaline hydrolysis for the preparation of samples has been criticized by Wooley and Sebrell (11) who found that, under the conditions they employed, sodium hydroxide hydrolysis was unsatisfactory for the preparation of samples for the microbiological determination of tryptophan. Kratzer (12) found that in some instances sodium hydroxide was unsatisfactory for the preparation of samples for the analyses of tryptophan by Eckert's method (13). Hess and Sullivan (14) apparently have successfully used sodium hydroxide hydrolysis. It appears that the conditions for satisfactory alkaline hydrolysis are quite critical but have been satisfactorily established by several investigators. It is interesting that the tryptophan content of casein as determined by Wooley and Sebrell (11) and Greene and Black (10) after enzyme hydrolysis; by Greene and Black (10), Kratzer (12) and the writers after barium hydroxide hydrolysis; and by Hess and Sullivan (14) after sodium hydroxide hydrolysis are in excellent agreement. Recovery experiments indicated the tyrosine was also racemized by barium hydroxide hydrolysis and that a further 10% loss occurred. Values reported in this paper for tyrosine are corrected for both the racemization and hydrolysis loss. The tyrosine values are the only ones to which a correction factor was applied. Preliminary investigations with 20% hydrochloric acid hydrolyzates suggest that these are unsatisfactory for tyrosine assays as the results are much too low. One trial suggested that, if phenylalanine results obtained with the barium hydroxide hydrolysate were corrected for racemization and an eight % hydrolysis loss, they compare favorably with those secured by acid hydrolysis. Average results for phenylalanine, with alkaline and acid hydrolyzates, expressed as *per cent* of the protein were as follows; for dry skim milk



4.5 and 4.5, for dry whole milk 4.6 and 4.6, for evaporated milk 4.1 and 4.2, for fresh milk 4.4 and 4.5 and for casein 4.8 and 5.2. Six samples of each type of milk and four samples of casein were hydrolyzed by each method.

The methods of hydrolysis used were carefully selected and certain checks of their suitability for this investigation were made as noted above, but it has not been possible to prove in each case that the amino acid content of the hydrolysate was equivalent to that in the intact protein. The satisfactory recovery of added free amino acid exposed to the hydrolysis conditions does not exclude its destruction or racemization either during or prior to the actual hydrolysis. The problem of amino acid destruction during hydrolysis, particularly tyrosine, tryptophan, threonine and methionine has been discussed by Martin and Syngé (15), Block and Bolling (1), Lugg (16) and Brand and Kassel (17).

#### MEDIA USED FOR VARIOUS AMINO ACIDS

For the valine and isoleucine assays with *L. arabinosus* the medium proposed by Schweigert, McIntire, Elvehjem and Strong (18) was used. The medium proposed by Wooley and Sebrell (11) was used for the tryptophan assays. The medium proposed by Dunn, Shankman, Camien, Frankl and Rockland (6) was used for assays with *Leuconostoc mesenteroides* and that proposed by Stokes, Gunness, Dwyer and Caswell (9) for assays with *S. faecalis*. Preliminary trials and recovery experiments indicated that the media and procedures were satisfactory for assays on both milk and casein hydrolysates.

#### STANDARDS

Standards were chosen from the best sources available. Whenever possible, samples of known purity and nitrogen content were chosen and samples from two or more sources were obtained. The microbiological activity of samples from two or more sources of leucine, valine, threonine, tryptophan and phenylalanine, respectively, were compared and found to be equal. One sample of isoleucine was slightly more active than the other and was chosen for the standard. *dl*-Threonine and *dl*-lysine were checked against *l*-threonine and *l*-lysine and the *dl*-forms proved to be one-half as active as the *l* forms. The *l* forms of leucine, histidine, tyrosine and tryptophan and the *dl*-forms of isoleucine, valine, threonine, lysine, phenylalanine and methionine were used as standards.

#### SAMPLES

Four samples of casein were assayed. Two casein samples were commercial vitamin-free samples, one sample was a commercial sample marked "acid washed" and one sample was prepared from fresh skim milk according to Cohn's method as given by Schmidt (19). Moisture, ash and nitrogen analyses indicated that the principal non-protein impurity in the samples was moisture. One sample apparently contained a small amount of another unidentified impurity. The six dry skim milk samples were equally distributed from three different plants. Four of the dry whole milk samples were from the same processing plant, the other two were from different plants. The evaporated milk samples were obtained from six different areas in the United States.

## RESULTS AND DISCUSSION

The average results of the amino acid assays and the range of the data from individual samples are compiled in Table I.

TABLE I  
*Essential Amino Acid Content of Milk*

	Dry Skim Milk		Dry Whole Milk		Evaporated Milk		Fresh Milk		Casein	
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
(Expressed as per cent of the milk)										
Arginine*	1.1	1.0-1.2	0.9	0.8-1.0	0.22	0.21-0.22	0.10	0.09-0.12	3.4	3.1-3.5
Histidine	0.89	0.79-1.06	0.61	0.57-0.64	0.14	0.13-0.15	0.07	0.06-0.08	2.44	2.30-2.5
Isoleucine	2.2	2.11-2.38	1.6	1.5-1.7	0.43	0.41-0.46	0.20	0.17-0.23	5.9	5.7-6.0
Leucine	3.9	3.7-4.1	3.0	2.6-3.1	0.73	0.71-0.77	0.34	0.31-0.36	9.2	8.7-9.5
Lysine	3.1	2.6-3.6	2.0	1.9-2.2	0.53	0.47-0.58	0.28	0.24-0.34	7.7	7.1-8.3
Methionine	0.78	0.58-0.98	0.54	0.48-0.61	0.15	0.13-0.18	0.06	0.05-0.08	2.5	2.4-2.6
Phenylalanine	1.6	1.5-1.8	1.2	1.1-1.3	0.29	0.27-0.31	0.14	0.12-0.15	4.8	4.6-5.0
Threonine	1.7	1.5-1.8	1.2	1.2-1.3	0.32	0.30-0.33	0.14	0.13-0.15	3.9	3.8-4.0
Tryptophan	0.52	0.49-0.55	0.35	0.28-0.38	0.084	0.080-0.090	0.043	0.040-0.049	1.09	1.05-1.15
Tyrosine**	1.9	1.8-2.2	1.4	1.2-1.5	0.38	0.32-0.37	0.17	0.16-0.18	5.3	4.6-5.8
Valine	2.1	2.0-2.4	1.6	1.5-1.6	0.46	0.42-0.50	0.20	0.19-0.22	5.8	5.4-6.0
(Expressed as per cent of the protein (N $\times$ 6.38))										
Arginine*	3.1	2.9-3.4	3.5	3.2-3.8	3.4	3.3-3.5	3.4	3.1-3.6	3.6	3.5-3.7
Histidine	2.4	2.2-2.9	2.4	2.4-2.5	2.0	1.9-2.1	2.3	2.1-2.8	2.6	2.6-2.7
Isoleucine	6.0	5.9-6.2	6.5	6.3-6.8	6.2	5.9-6.4	6.3	6.2-6.5	6.4	6.2-6.4
Leucine	10.6	9.9-11.1	11.3	11.0-12.2	11.2	10.6-11.5	10.9	9.9-11.9	9.9	9.8-10.0
Lysine	8.3	7.5-9.1	8.1	7.8-8.7	7.4	7.2-7.7	8.0	8.3-9.2	8.3	8.1-8.8
Methionine	2.1	1.6-2.6	2.2	2.0-2.5	2.2	2.1-2.4	1.9	1.7-2.1	2.6	2.1-2.8
Phenylalanine	4.5	4.2-5.0	4.6	4.4-5.0	4.2	3.7-4.5	4.5	4.3-4.7	5.2	5.0-5.3
Threonine	4.5	4.2-4.9	4.8	4.7-4.9	4.9	4.6-5.1	4.6	4.2-4.7	4.2	4.1-4.3
Tryptophan	1.4	1.2-1.5	1.4	1.1-1.5	1.2	1.2-1.3	1.4	1.2-1.4	1.2	1.1-1.2
Tyrosine**	5.3	4.9-5.5	5.5	4.7-5.7	4.9	4.7-5.2	5.3	5.1-5.7	5.7	5.1-6.0
Valine	5.9	5.5-6.6	6.2	6.0-6.5	6.2	6.0-6.7	6.3	6.0-6.5	6.2	6.1-6.6

\* Arginine is required only for maximum growth by certain species. It cannot be considered truly indispensable.

\*\* Tyrosine is not an indispensable amino acid, but it may exert a sparing action on phenylalanine.

For comparative data the reader is referred to the book by Block and Bolling(1) and the paper by Williamson (2). The data presented in these references were obtained largely by chemical methods. Stokes, Gunness, Dwyer and Caswell (9) present data obtained by their microbiological method on a sample of whole milk and one of casein. In most cases, the data presented in the latter paper are in good agreement with those reported in Table I.

The data are expressed as *per cent* of the sample (undried basis) and as *per cent* of the protein (micro-kjeldahl nitrogen multiplied by 6.38). In this calculation of protein from the nitrogen content, much of the error inherent in the use of a common factor for all proteins (6.25) is eliminated by use of a specific factor for casein. Never-

theless, a small error may still exist as the factor 6.38 may not be accurate for all the milk proteins or for all variations of protein content that may occur in the samples under consideration. However, the calculated protein value is extremely useful for comparative purposes, as it reduces to an approximately equivalent basis, different kinds of food or dissimilar samples of the same food. In this investigation the calculated protein value is especially useful in comparing the samples of fresh milk in which the protein content varies considerably, and in making comparisons between the different types of milk as these may vary in fat (dry skim milk) as well as protein and are of widely different moisture content. The data include the range of values as well as the average in order to convey the extent of variability in results of the assays. Because of the small number of samples involved for each type of milk, it seemed preferable to express this variability in terms of the range instead of standard deviation. On a comparable protein basis, the results for arginine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, tyrosine and valine, with a few minor exceptions, fall within  $\pm 10\%$  of the average. A number of factors, the duplicability of the hydrolyzates, duplicability of titrations in colored solutions, nitrogen analyses, as well as variability of bacterial growth, combine to give an overall variability in results that may approach  $10\%$ . For this reason, it appears probable that the apparent variability on a given type of milk may be chiefly assigned to the method rather than to actual differences between samples. Whether or not the somewhat greater differences between the histidine values and between the methionine values for certain samples have any significance is doubtful because the methods for these two amino acids were somewhat more troublesome than those for the other amino acids. It should not be inferred from our work that the overall variability of all microbiological amino acid assays is  $\pm 10\%$ . The variability can be decreased by the use of a larger number of replicates and is usually less for purified and semi-purified proteins. In general, the variability in results reported in Table I for casein are not as great as those reported for the various types of milk.

In most instances the values in Table I confirm published data. Where this is the case, they will not be discussed in detail unless there is an indication of a difference between fresh and processed milk. In the case of histidine, there appears to be a slightly lower content in evaporated milk. A later and more extensive investigation failed to confirm this point, and we are at present inclined to assign this lower average value for histidine to the variability of the method. It seemed probable that any such loss of histidine would occur in the heat sterilization of evaporated milk. However, in a comparison of four pairs of samples of unsterilized and sterilized evaporated milk, an average difference of only  $3\%$  of histidine was found and, when the processing time was increased fourfold, as compared to commercial practice, on two samples the loss amounted only to 8 and  $13\%$ .

Our leucine values are lower than the chemical values reported by Williamson (2) and by Block and Bolling (1). They are in much better

agreement with the microbiological values reported by Stokes, Gunness, Dwyer and Caswell (9), by Ryan and Brand (20) and by Schweigert, McIntire, Elvehjem and Strong (18). Our lysine values also agree much better with other published microbiological values than with the chemical values. While the data in Table I suggest a slight loss of lysine by the heat treatment in the preparation of dried milk, this is of doubtful significance because of the overlapping in the range of values for the various types of milk. The slightly greater loss in lysine in the preparation of evaporated milk suggested by the data appears to have some significance. In an attempt to confirm the accuracy of this observation, the lysine content of four samples of evaporated milk were compared before and after sterilization. These samples were sterilized for 15 minutes at temperatures from 116 to 118°C. in a continuous sterilizer. The average loss in lysine content was 11%. When samples were sterilized for one hour at the same temperature the average loss was 26%. The latter samples were so badly overcooked that they were unfit for human consumption. The conditions for the sterilization of evaporated milk are critical. The fluid character of milk permits agitation in the can to promote heat penetration and extensive commercial experience has shown that adherence to the conditions stated above insure the production of a sterile milk but permit stabilization of the protein in an uncoagulated form and minimize the production of cooked flavor.

Since the nature of the protein, the pH of the media and other conditions affect the rate of lysine destruction during sterilization, it would be unwise to project our data for evaporated milk to the estimation of lysine destruction in other canned foods. Nevertheless, our work suggests the need for an investigation of this subject in the case of meat and non-acid vegetable products which require long sterilization times which are often several-fold that which can be used for evaporated milk.

The destruction of lysine in the heat processing of proteins has been noted before. Morgan and Kern (21) found that cooking reduced the nutritive value of meat protein. Greaves, Morgan and Loveen (22) found that the dry heating of casein destroyed the lysine and that, at temperatures above 140°C., histidine was also destroyed. Waisman and Elvehjem (23) found that autoclaving edestin destroys lysine. The data presented in the papers mentioned are largely drawn from prolonged heat treatment at high temperatures which were sufficient

to alter the biological value of the protein as measured by nitrogen balance or growth-promoting properties. In contrast, since milk protein is one of the richest sources of lysine, the loss during the processing of evaporated milk is small and does not detract from the value of the protein when milk is the only or major source of protein in the diet as it is in the determination of biological value of the protein or in an infant formula. This has been shown by Whitnah (24) who found that the biological value of evaporated milk protein as determined by the Mitchell method does not differ significantly from that of fresh milk. Greaves, Morgan and Loveen (22) have explained that the low content of cystine and methionine is the factor limiting growth in studies with milk protein and that sufficient excess lysine is present so that a part of it may be destroyed before the loss is reflected in the biological value of the protein. While the supplement value of milk proteins when combined with other proteins deficient only in lysine may be less in the case of evaporated milk, the loss of lysine is so small that it is of doubtful practical significance.

The methionine content of milk and casein as reported in Table I are in good agreement with those reported by Stokes, Gunness, Dwyer and Caswell (9) but are somewhat lower than those reported by Block and Bolling (1), which were obtained by chemical methods. The values for methionine in casein reported by Evans (25) and by Albanese, Frankston and Irby (26) and obtained by two different chemical methods are also in good agreement with those reported in Table I. The lower methionine content in mixed milk protein than that in casein is unexplained. We have investigated the question of whether the presence of lactose in the hydrolysis mixture contributes to the loss of methionine. When methionine was added to a lactose-casein mixture, recoveries of only 90% were obtained, but when methionine was added to dry skim milk excellent recoveries were obtained. While it is not possible to draw a definite conclusion as to whether there may or may not be a small loss of methionine, it does appear that no very large loss occurs when milk proteins are hydrolyzed in the presence of lactose. We have used the method of Stokes, Gunness, Dwyer and Caswell (9) to check our methionine results on the dry skim milk samples. An average result of 2.1% of the protein was obtained by this *S. faecalis* method which is the same as that obtained by the *Leuconostoc mesenteroides* method. Somewhat less variation was obtained by the *S. faecalis* method, but the agreement between results

on the individual samples was quite good. Two samples of casein were also assayed by both methods and the results are in excellent agreement. In our hands, the *S. faecalis* method of Stokes, Gunness, Dwyer and Caswell (9), which we used for arginine, leucine, threonine and methionine assays, gives somewhat more uniform results than those employing other bacteria.

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#### SUMMARY

1. Microbiological methods have been used to assay six samples each of fresh, evaporated, dry whole and dry skim milk and four samples of casein for the nine essential amino acids, arginine and tyrosine.

2. With the exception of slightly lower values for leucine and slightly higher values for lysine, the data on amino acid content do not differ markedly from published values obtained by chemical methods and the agreement with other microbiological data is good.

3. The data suggest a small and nutritionally insignificant loss of lysine in the heat processing of evaporated milk. When the variability of the assays is considered, this is the only measurable indication of a loss of an essential amino acid during the processing of evaporated or dried milk.

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## Studies on the Nutritional Requirements of *Bacillus anthracis*<sup>1</sup>

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### INTRODUCTION

The nutritional requirements of *Bacillus anthracis* have received relatively little attention. The only detailed published work is that by Gladstone (1939). He grew several strains of *B. anthracis* in "completely synthetic" media consisting of amino acids, salts, glucose and glutamine; no mention was made of any vitamin requirement. Information was presented on the necessity for having several amino acids in the medium, and on the interrelationships between them. Fildes and Gladstone (1939), using Gladstone's "synthetic" medium, found that the growth of five of six strains of *B. anthracis* was accelerated by the addition of M/1000 glutamine, whereas the growth of the other strain was unaffected. Knight (1935) reported that a concentrate of his "staphylococcus factor" from yeast was essential for the proper growth of a strain of *B. anthracis*. The "staphylococcus factor" was later identified as nicotinic acid and thiamine (Knight, 1937), but Landy (1939) reported that the essential growth factor for *B. anthracis* was neither of these vitamins, but still another factor, not replaceable by any of the known growth factors. O'Kane (1943) found that thiamine was the only B complex vitamin required by *B. anthracis* in a hydrolyzed casein base medium.

The work presented here is the result of studies on the nutritional requirements of *B. anthracis*, Vollum strain,<sup>3</sup> in a completely chemically

<sup>1</sup> Studies conducted at Camp Detrick, Frederick, Md., from August, 1944, to February, 1945.

<sup>2</sup> With the technical assistance of M/Sgt. H. J. Buehler, T/5 W. L. Mosby and PhM3c J. M. Heinzman.

<sup>3</sup> Vollum, M-36 strain obtained from Dr. Paul Fildes.



After moderate growth had been obtained in acid-hydrolyzed casein base media, chemically defined media were devised on the basis of the results with semi-pure media.

TABLE II

*The Effects of Ammonium Sulfate, Ammonium Nitrate, Urea and Yeast Nucleic Acid on Growth of B. anthracis at Various Levels of Casein Hydrolyzate*

No.	Constituents added to basal medium*	Per cent casein hydrolyzate		
		0.2	0.4	0.6
		Spores $\times 10^6$ /ml.		
1	None	27	40	41
2	0.2% ammonium sulfate	32	35	39
3	0.2% ammonium nitrate	25	41	48
4	0.2% urea	35	35	45
5	0.1% yeast nucleic acid	79	152	249

\* Basal medium: 0.015 M  $\text{KH}_2\text{PO}_4$ , 0.015 M  $\text{K}_2\text{HPO}_4$ , 0.0002 M  $\text{MgSO}_4$ , 0.00004 M  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ , 0.025 M  $\text{NaHCO}_3$  and 0.02  $\gamma$ /ml. thiamine-HCl.

Several mixtures of pure amino acids (synthetic where possible) were used in preliminary attempts to replace the casein hydrolyzate. By coincidence, the mixture devised to resemble casein (on the basis of published amino acid analyses) gave the best results of those tested. Reducing the concentrations of several amino acids and increasing those of others, improved growth further. The best combination developed in the early experiments ("D" mixture) is shown in Table III. This mixture is not ideal; some of its components are unnecessary for growth and the concentration of others might profitably be altered. It was later found, after the inorganic composition of the basal medium had been improved, that increasing the level of the "D" mixture in the medium allowed still better growth (Fig. 1). The upper set of amino acid data in the figure was obtained in a later experiment with a further improved basal medium.

Several experiments were carried out to study the effects of individual amino acids on growth. It was soon evident that an extensive program would be necessary to determine the requirement for each amino acid in the presence of optimal amounts of all the others, in addition to various other interrelationships between them. Because of time limitations, no further work was done on amino acid requirements.

TABLE III  
Amino Acid Mixture ("D" Mixture)

Amino acid	Final molarity in medium
Glycine	0.001
dl-Alanine	0.0025
dl-Serine	0.0005
dl-Threonine	0.0025
dl-Valine	0.00125
dl-Leucine	0.0025
dl-Isoleucine	0.0025
dl-Aspartic Acid	0.00125
dl-Glutamic Acid	0.0025
dl-Lysine · HCl	0.0005
l(+)-Arginine · HCl	0.00125
l(+)-Histidine · HCl	0.00125
l(-)-Cystine	0.000125
dl-Methionine	0.0005
l(-)-Proline	0.0025
dl-Phenylalanine	0.001
l(-)-Tryptophane	0.000625
l(-)-Tyrosine	0.0005

Note: "2D" mixture contains twice the above concentrations of amino acids, except that tyrosine is omitted.

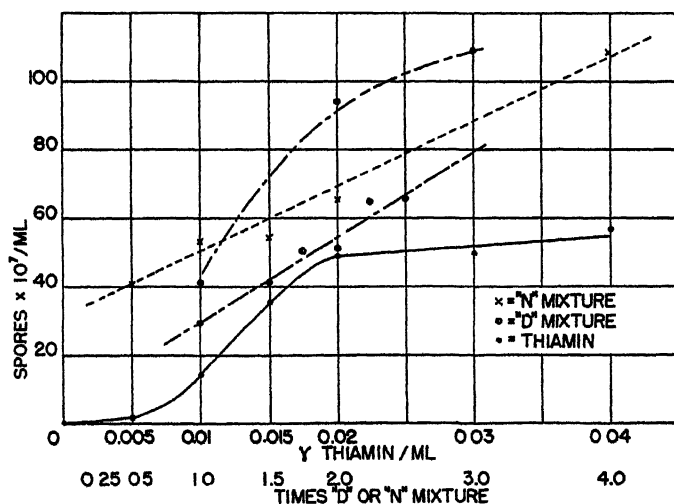


Fig. 1

Effect of "N" Mixture, "D" Mixture and Thiamine on Growth of *B. anthracis* in Chemically Defined Media

After an adequate amino acid mixture was obtained, the next step was to study the replacement of the yeast nucleic acid with known compounds. Of the known components of ribonucleic acid, uracil, adenine, guanine and ribose were combined in the same relative concentrations in which they occur in ribonucleic acid, and at levels at which they had been found effective with other bacteria. The resulting mixture of nucleic acid components ("N" mixture) was equivalent in concentration to 0.00005 *M* ribonucleic acid. The results indicate that the nucleic acid is completely replaceable by "N" mixture. The effects of increasing the levels of "N" mixture are shown in Fig. 1.

Table IV shows the effect on growth of single constituents of the "N" mixture as well as various combinations of those constituents.

TABLE IV  
*Effect of Constituents of Nucleic Acid\* on Growth of B. anthracis  
in a Chemically Defined Medium*

Medium	Spores $\times 10^7$ /ml.
Basal (As Table VI without "N" mixture)	41
Basal + "N" mixture	78
Basal + Uracil	42
Basal + Adenine	49
Basal + Guanine	30
Basal + Ribose	28
Basal + Uracil + Adenine	80
Basal + Uracil + Guanine	63
Basal + Uracil + Ribose	39
Basal + Adenine + Guanine	62
Basal + Adenine + Ribose	50
Basal + Guanine + Ribose	44
Basal + Uracil + Adenine + Guanine	75
Basal + Uracil + Adenine + Ribose	60
Basal + Adenine + Guanine + Ribose	67
Basal + Uracil + Guanine + Ribose	37
Basal (As Table VI without "N" mixture)	39
Basal + "N" mixture	53
Basal + 2 "N" mixture	65
Basal + 4 "N" mixture	109
Basal + Uracil + Adenine	51
Basal + 2 Uracil + 2 Adenine	34
Basal + 4 Uracil + 4 Adenine	40

\* Concentrations used are 0.00005 *M* uracil, adenine and guanine and 0.0002 *M* ribose, except where two or four times these concentrations are indicated.

It can be seen that none of the compounds was effective singly. At the single level of "N" mixture, a combination of uracil and adenine was as effective as the complete mixture, but at double and quadruple levels growth was diminished. At higher levels of "N" constituents, which resulted in better growth, guanine was needed in addition to uracil and adenine. Combinations of uracil and guanine or adenine and guanine were partially effective. Ribose was not needed and, in several cases, its inclusion in the medium resulted in lower yields. Other experiments indicated that the addition of cytosine or thymine to the mixture did not further stimulate growth, that animal nucleic acid functioned fully as well as yeast nucleic acid, as would be expected, and that adenosine or adenylic acid successfully replaced adenine but did not improve growth beyond that obtained with adenine.

The following B complex vitamins, in the concentrations indicated ( $\gamma$ /ml.), gave little or no stimulation of growth when added singly or combined to a purified basal medium containing thiamine: riboflavin 0.1, pyridoxine 2.0, calcium pantothenate 1.0, niacinamide 1.0, biotin 0.001, *p*-aminobenzoic acid 0.005, inositol 20.0, and *L. casei* factor 0.01. However, the vitamin mixture was added in most experiments as a precaution because of its possible effects on virulence or stability. Thiamine is the only vitamin needed by this organism.

The optimal level of thiamine was determined on a purified medium, containing amino acids and the "N" mixture in place of hydrolyzed casein and nucleic acid. Fig. 1 shows the curve obtained. Thiamine levels of 0.02  $\gamma$ /ml. or higher allowed maximum growth. The pyrimidine and thiazole components of thiamine were found to be inactive, both singly and in combination.

The modified Gladstone salt mixture (Table I, footnote) had been adequate for early work on organic nutrients, but it was realized that it could be improved. When the completely defined medium was available, the mineral nutrition of the organism was studied.

Table V shows the results obtained with added  $Mg^{++}$ ,  $Ca^{++}$ ,  $Fe^{++}$  and  $Mn^{++}$ . In this experiment each ion was tested individually by varying its concentration while the other ions were present in approximately optimal concentration as determined in preliminary experiments. In the absence of magnesium practically no growth occurred, whereas 0.0005 *M*  $MgSO_4$  allowed good growth. A yield of  $10 \times 10^7$  spores/ml. was obtained in the absence of calcium, but the addition of 0.0005 *M*  $CaCl_2$  increased the yield to  $45 \times 10^7$  spores/ml. Higher

TABLE V

*Effect of Metallic Ions on Growth of B. anthracis in Chemically Defined Media*

Metallic ion varied	Molarity of variable	Yield Spores $\times 10^7$ /ml.
Ca <sup>++</sup> (Fe, Mg present)	0	11
Ca <sup>++</sup> (Fe, Mg present)	0.0001	40
Ca <sup>++</sup> (Fe, Mg present)	0.0005	44
Ca <sup>++</sup> (Fe, Mg present)	0.0025	20
Ca <sup>++</sup> (Fe, Mg present)	0.01	<1
Fe <sup>++</sup> (Ca, Mg present)	0	17
Fe <sup>++</sup> (Ca, Mg present)	0.000013	46
Fe <sup>++</sup> (Ca, Mg present)	0.00004	40
Fe <sup>++</sup> (Ca, Mg present)	0.0002	43
Fe <sup>++</sup> (Ca, Mg present)	0.001	40
Mg <sup>++</sup> (Fe, Ca present)	0	<1
Mg <sup>++</sup> (Fe, Ca present)	0.000067	38
Mg <sup>++</sup> (Fe, Ca present)	0.00067	34
Mg <sup>++</sup> (Fe, Ca present)	0.00134	42
Mn <sup>++</sup> (Fe, Ca, Mg present)	0	44
Mn <sup>++</sup> (Fe, Ca, Mg present)	0.00001	51
Mn <sup>++</sup> (Fe, Ca, Mg present)	0.0001	50
Mn <sup>++</sup> (Fe, Ca, Mg present)	0.001	56

Basal medium: "D" mixture (Table III), "N" mixture, 0.02 *M* glucose, 0.02 *M* NaHCO<sub>3</sub>, 0.00003 *M* glutamine, 0.03 *M* K<sub>2</sub>HPO<sub>4</sub>, 0.03 *M* KH<sub>2</sub>PO<sub>4</sub>, 0.02  $\gamma$  thiamine ·HCl/ml., and the mixture of eight B complex vitamins described in the text.

levels of calcium were toxic until at 0.01 *M* no growth occurred. Levels of iron (as FeSO<sub>4</sub>) from 0.00001 *M* to 0.001 *M* all gave equal growth whereas, in the absence of iron, growth was markedly reduced. Added manganese (as MnSO<sub>4</sub>) was not essential, but stimulated growth appreciably. It is probable that the medium contained enough manganese as an impurity to allow moderate growth. Some of the other essential elements were also undoubtedly present as contaminants.

Replacement of the potassium phosphates in the medium with sodium phosphates resulted in a decrease in yield from  $35 \times 10^7$  spores/ml. to  $10 \times 10^7$  spores/ml. Partial replacement reduced the yields proportionately.

The optimal level of total phosphate ion was found not to be critical. In early work 0.03 *M* and 0.06 *M* phosphate gave equal yields, whereas

in later improved media 0.06 *M* phosphate gave somewhat better yields than lower concentrations; 0.09 *M* phosphate was definitely toxic.

The addition of  $\text{CuSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{CdCl}_2$  and  $\text{CoCl}_2$  at levels of 0.1 to 1.0 ppm. of the metallic ions caused no stimulation of growth, and 10 ppm.  $\text{Cu}^{++}$  was definitely toxic.

In a separate experiment on the effects of possible traces of other inorganic ions in the medium, the components of the medium (amino acids, salts, glucose, vitamins, etc.) were purified individually by appropriate treatment with diphenylthiocarbazone or 8-hydroxyquinoline or, in some cases, by repeated recrystallization. The results showed that the fully purified medium was somewhat less satisfactory for growth than the non-purified medium. The difference is probably due to removal of traces of some element or elements helpful to growth, although the possibility of residual toxicity or of damage to organic nutrients by the method of treatment must not be overlooked. Intermediate combinations gave intermediate values. This phase of the work was not carried further.

TABLE VI  
"Synthetic" Medium for *B. anthracis*

Compound	Molarity
<i>d</i> (+)-Glucose	0.02
<i>l</i> (+)-Glutamine	0.00003
2 × "D" mixture (Amino Acids)	—
4 × "N" mixture (Nucleic Acid Components)	—
$\text{NaHCO}_3$	0.02
$\text{K}_2\text{HPO}_4$	0.03
$\text{KH}_2\text{PO}_4$	0.03
$\text{MgSO}_4$	0.0002
$\text{MnSO}_4$	0.0001
$\text{FeSO}_4$	0.00005
$\text{CaCl}_2$	0.0005
Thiamine·HCl	0.0000006

The composition of the best chemically defined medium for *B. anthracis*, based on the results of these studies, is shown in Table VI. This medium produced yields in excess of one billion spores/ml., and controlled increases in glucose, amino acids and nucleic acid components would probably increase the yield still further.

Spores grown in this chemically defined medium were extremely virulent. When injected subcutaneously five spores killed an average of three out of five mice, while 20 spores killed 100% of the mice, with symptoms and pathology of typical anthrax.

## DISCUSSION

The vitamin requirements of this strain of *B. anthracis* are relatively simple, thiamine being the only vitamin required. Uracil, adenine and guanine are necessary for optimal growth. Gladstone's results with a medium supposedly free from these factors may be due to strain differences, or he may have obtained less growth than we did. The failure of Landy to identify thiamine as one of the required factors may be due to the fact that both thiamine and the nucleic acid components must be present for appreciable growth; with either absent, growth is very light. The requirement of the organism for uracil, adenine and guanine is not surprising, as several other organisms have been shown to require one or more purine or pyrimidine bases (Pappenheimer and Hottle, 1940; Snell and Mitchell, 1941; Mueller and Miller, 1942; Hutner, 1944).

The striking effects of various metallic ions on this organism's growth serve to emphasize the importance of inorganic nutrition for microorganisms; it is a factor often overlooked or minimized.

Bicarbonate (or CO<sub>2</sub>) promoted earlier spore germination. Kempner and Schlager (1942) have shown that with the *pneumococci* the length of the lag phase is an inverse function of the CO<sub>2</sub> concentration.

It should be remembered in examining these data that some of the experiments are not comparable with others, since they were performed at different stages of the work, with corresponding differences in yields to be expected. Certain aspects of this work, such as the interrelationships between the amino acids and between the nucleic acid components, are admittedly incomplete and should receive further study. This, however, was not possible under war conditions because of the urgent need for application of these results in the solution of practical problems.

## SUMMARY

1. The nutritional requirements of a strain of *B. anthracis* have been determined.

2. Yields in excess of  $10^9$  viable spores/ml. have been obtained in a chemically defined medium consisting of amino acids, glucose, glutamine, bicarbonate, salts, thiamine, uracil, adenine and guanine.

3. The only vitamin required by *B. anthracis* is thiamine; uracil, adenine and guanine are stimulatory.

4. Calcium, magnesium, iron, manganese, potassium, bicarbonate and phosphate are either required for, or stimulate, growth.

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# Application of Nutritional Studies for Development of Practical Culture Media for *Bacillus anthracis*<sup>1</sup>

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## INTRODUCTION

In order to develop an improved practical growth medium specifically for *Bacillus anthracis*, the results of nutritional studies (Brewer *et al.* 1946) with this species were applied to the selection of appropriate nutrient materials commercially available at moderate cost, and to the proper combination of these materials to form a satisfactory medium of relatively low solids content. The previous work had shown that *B. anthracis* required for satisfactory growth a number of the natural amino acids (in proportions similar to those in casein), glucose, thiamine, adenine, guanine, uracil, bicarbonate, phosphate and salts of potassium, magnesium, iron, manganese and calcium.

## EXPERIMENTAL

The experimental techniques described in the fundamental nutritional studies with *B. anthracis* were used in devising practical growth media. After preliminary work with 15 ml. cultures had indicated suitable combinations, these were tested and confirmed on a larger scale by growth in volumes of 500 ml. or larger in 4 liter Pyrex Fernbach flasks on the shaking machine previously described. It was found desirable to clarify by filtration or centrifugation the undried distillers' solubles to remove insoluble fibrous grain residues.

## RESULTS

Since *B. anthracis* had been shown in the work with hydrolyzed casein and with chemically defined media to require several amino

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<sup>1</sup> Work conducted at Camp Detrick, Frederick, Md., from November, 1944, to March, 1945.

<sup>2</sup> With the technical assistance of M/Sgt. H. J. Buehler, T/5 W. L. Mosby and PhM3c J. M. Heinzman.

acids for growth, it was apparent that a protein hydrolyzate should be included in the medium. The most suitable of those available was peptidase,<sup>3</sup> a tryptic digest of casein. Because casein is more uniform in composition than the packing house wastes ordinarily used in "peptones," because it is a complete protein and because its acid hydrolyzate had served satisfactorily for growth of *B. anthracis*, peptidase was selected for initial trial as a source of amino acids.

The necessity for the components of nucleic acid in the medium indicated the desirability of including yeast or yeast products. Yeast also serves as a good source of thiamine, an essential nutrient.

The remaining nutritional requirements, fermentable carbohydrate and the inorganic ions bicarbonate, phosphate, potassium, magnesium, manganese, iron and calcium can be obtained cheaply and in large quantities as commercially pure substances. Moreover, the enzyme digest of casein and the yeast materials used can provide a part of these requirements, and in some cases, *e.g.*, bicarbonate and thiamine, all of that required.

Accordingly, the early attempts to devise practical media were carried out with combinations of peptidase, marmite (a British yeast autolyzate) or undried distillers' solubles (semi-soluble residue of the alcoholic fermentation of grain), glucose and salts. Many combinations of these ingredients were tried with success.

Although peptidase was considered to be the most satisfactory of the commercial protein digests, others were also tested to determine their suitability for use in alternate media. These alternative digests included several brands of peptones. They were tested at several concentration levels both singly and in combination.

Several media which have produced yields of approximately two billion or more spores per ml. are listed in Table I. The level of any particular protein digest or yeast product was found to be non-critical, but rather a minimum total concentration of each of these appeared to be required for high yields. Within limits, the various protein digests were mutually replaceable as were the yeast products.

## DISCUSSION

Complex media of unknown and variable composition (*e.g.*, meat infusions, serums, *etc.*) are usually employed for the cultivation of

<sup>3</sup> A product of Sheffield Farms Co., Inc., New York, N. Y.

pathogenic bacteria, particularly when cultures of larger than test tube size are required. Such empirical media are seldom fitted to the specific nutritional needs of the individual species and may often be nutritionally deficient. Unnecessary proteins thus included in the medium may also introduce needless complications in immunological studies in which such media are used.

TABLE I  
*Practical Media for Bacillus anthracis*

No.	per cent Pepti- case <sup>1</sup>	per cent USP Peptone <sup>2</sup>	per cent DS <sup>3</sup>	per cent Mar- mite <sup>4</sup>	per cent PY <sup>5</sup>	per cent Cereulose	per cent K <sub>2</sub> HPO <sub>4</sub>	per cent KH <sub>2</sub> PO <sub>4</sub>	Spores × 10 <sup>7</sup> /ml.
1	1.0			0.075		0.4	0.33	0.27	150 <sup>6</sup>
2	1.0		0.6			0.8	0.5	0.4	195
3	1.0		1.0			0.6	0.5	0.4	195
4	1.0		0.8	0.1		0.8	0.5	0.4	204
5	0.6	0.6	0.4			0.8	0.5	0.4	198
6	0.6	0.6	0.6			0.8	0.5	0.4	222
7	1.0	1.0	0.5		0.1	0.8	0.5	0.4	226

Basal salts: 0.01% CaCl<sub>2</sub>·6H<sub>2</sub>O, 0.001% FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.005% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.003% MnSO<sub>4</sub>·4H<sub>2</sub>O, tap or distilled water to volume.

1. Product of Sheffield Farms Co., Inc., New York, N. Y.
2. Product of The Wilson Laboratories, Chicago, Ill.
3. Undried distillers' solubles from Hiram Walker and Sons, Inc., Peoria, Ill.
4. A British autolyzed yeast product.
5. Plasmolyzed yeast from Vico Products Co., Inc., Chicago, Ill.
6. Contained 0.02γ thiamine per ml. and 0.02 M NaHCO<sub>3</sub>.

One of the media described (No. 1, Table 1) in this paper has proven of value for the production of large yields of highly virulent spores in investigations of the immunochemistry of *B. anthracis* by other workers (Watson *et al.* 1946). These workers found that virulence was maintained by continuous cultivation in pepticase-marmite media but attenuation of virulence resulted from growth on nutrient agar. A similar medium has been used (Olson, 1946) with success for the preparation of spore suspensions of *Bacillus globigii* for experiments with electro-physical methods of counting bacteria, where freedom of the medium from non-bacterial microparticles was required.

## SUMMARY

By the application of the results of previous nutritional studies, practical media designed specifically for growth of *B. anthracis* have been developed.

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# On the Respiration of Dermatophytes

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## INTRODUCTION

There does not seem to be any report on the respiration of members of the dermatophytes (species of the filamentous genera of *Imperfecti*: *Trichophyton*, *Epidermophyton* and *Microsporum* causing superficial infections of the skin). The literature to be cited, although based mainly on members of the Phycomycetes, has some bearing on our problem. The extensive work by De Boer (1928) on the respiration of *Phycomyces* included a critical discussion of much of the older pertinent literature.

De Boer found *Phycomyces Blakesleeanus* could not grow anaerobically and was affected by even a slight drop in oxygen tension. On linseed oil the fungus had an R.Q. of 0.65-0.75; fat was metabolized preferentially in mixed carbohydrate-fat substrates. Butkewitsch (1902) had shown certain fungi only consume proteins when carbohydrates or fats were not present; in the absence of the latter compounds *Aspergillus niger* liberated considerable quantities of ammonia while *Penicillium* sp. liberated amino acids. Schade and Thimann (1940) investigated oxygen consumption by a water mold, *Leptomitius lacteus*. They found *l*-leucine or *dl*-alanine capable of supporting growth as the sole source of carbon and nitrogen; these amino acids were found to be oxidized by mycelia that had been depleted of reserve stores by standing several days in a nonnutrient environment. With a source of nitrogen present, acetate,<sup>3</sup> butyrate, and higher fatty acids up to 10 carbon atoms were oxidized;

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<sup>3</sup> It is possible that *Leptomitius* might be a most favorable organism for the study of acetate oxidation inasmuch as dextrose is not oxidized and the oxidation of acetate exhibits a partial recovery with time from inhibition by cyanide; Nickerson and Carroll (1945) have presented a brief discussion of differences among organisms in the oxidation of acetate.

under similar conditions dextrose or sucrose were not oxidized. The essentially assimilative nature of this organism was shown by the fact that no oxygen uptake in excess of the control was found with unstarved mycelia upon the addition of any carbon source.

Robbins and Ma (1945), studying the nutrition of *Trichophyton mentagrophytes*, found this organism unable to use  $\text{NH}_4\text{NO}_3$  but to grow readily with a source of amino acids (i.e., peptone, casein hydrolyzate). They found no evidence for the indispensability of any amino acid but no single amino acid promoted growth so well as did a suitable mixture of amino acids. The authors concluded that amino acids available in a medium are incorporated into the fungus protein more rapidly than these can be supplied by the metabolic transformation of any single amino acid.

In the present study on oxygen uptake by species of dermatophytes under various experimental conditions, respiratory data emphasize the role of assimilative processes in these organisms. Through variations in the rates of oxygen consumption prompted by changes in hydrogen ion and salt concentrations, some considerations of the nature and location of respiratory enzymes within the cell are put forth. Additions of minute amounts of various cations and selected organic compounds to respiring fungus preparations revealed several substances with inhibitory effects on oxygen uptake; efforts were made to neutralize the inhibitory effects of some of the cations. A suggestion is put forth on the use of respiratory measurements in the bioassay of fungicides.

## EXPERIMENTAL

### Methods

*A. Organisms.* Cultures of pathogenic fungi freshly isolated<sup>4</sup> from lesions of the foot and of the glabrous skin were identified following Emmons (1934) and Conant *et al.* (1945). These cultures, and cultures received<sup>5</sup> from Duke University Medical School, were inoculated onto plates of Sabouraud's dextrose agar and Difco-cornmeal agar. Transfers were also inoculated into a liquid medium of the following composition: dextrose 50 g., tryptose 10 g., asparagine 1 g., yeast extract 0.01 g.,  $\text{K}_2\text{HPO}_4$  5 g.,  $\text{MgSO}_4$  0.2 g. and distilled water to make one liter; total solids, 6.63 g. per 100 cc. The following organisms were used: *Trichophyton gypseum* (mentagrophytes, cf. Emmons, 1934), *T. rubrum* and *Epidermophyton floccosum*.

*B. Fungus Preparations for Respirometer.* The filamentous fungi frequently grow in the form of a mat on the surface of agar media and, unless disturbed, grow similarly

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<sup>4</sup> Isolations were made by Capt. Frank A. Dolce, MC, in the Dermatological Clinic, AAF Regional Station Hospital, Eglin Field, Fla. A preliminary report by Dolce and Nickerson on the clinical use of dilute solutions of zinc chloride in the treatment of fungus infections among military personnel is in preparation.

<sup>5</sup> The kindness of Dr. N. F. Conant in supplying cultures is appreciated.

on the surface of liquid media. Attempts to harvest such growth and to homogenize it in some way (such as by sucking in and expelling from a syringe) for use as a suspension have been reported occasionally, but such techniques defeat the purpose—the mycelial components are frequently ruptured and one most often obtains merely a spore suspension with assorted cell debris (the admirable Klyver-Perquin agitation method for producing homogeneous growth was not entirely feasible because of the slow growth-rate of these fungi). It was our desire to learn to what extent a foreign agent effects a healthy, growing culture in a state of organization for *in vitro* study closely comparable to that *in situ*. To this end, using a sterile 15 mm. diameter cork borer, cylinders were cut from agar plate cultures evenly covered with a 2 to 4 weeks old growth. The discs were then placed in the respirometer vessel in 1 cc. of liquid. After each experimental period the disc was dropped into boiling water to remove the agar, lifted from the water after one minute, drained and placed on a previously weighed, clean coverslip. Dry weight determinations were made by heating the fungus and coverslip for 12 hours at 100°C. It was found that loss of weight by the fungus mycelium during the brief hot water extraction was probably negligible.<sup>6</sup> The coverslip and fungus were mounted in lacto-phenol cotton blue on a slide to give a permanent record of the organism used in each test. If contamination occurred it would be easily discovered with such a record. Samples of fungus from liquid cultures were easily obtained by use of a wire flattened at the end to a sharp cutting edge capable of being flamed. Pieces of mycelia were lifted out from a culture flask, drained, rinsed in sterile distilled water and placed in the respirometer vessel.

*C. Handling of Respirometer Vessels.* A volumetric microrespirometer described by Scholander (1942) and by Scholander and Edwards (1942) was used in the present work. This instrument, discussed by Glick (1944), employs an ordinary micrometer to displace mercury from the reservoir of a micrometer burette and has a sensitivity of 1/3 mm.<sup>3</sup> per hour. An important feature of this type of instrument is that the pressure of oxygen in the vessel is maintained constant during long experiments. Vessels were of 10 cc. capacity; CO<sub>2</sub> was absorbed by 20% KOH placed in a small well of which the upper half was paraffined. A filter paper roll (Whatman No. 40) saturated with KOH protruded slightly from the well. Care was taken to touch the paper only with clean forceps to preclude formation of ammonia.

A disc of fungus mycelium from an agar culture or a piece of mycelium of suitable size from a liquid culture was carefully lowered into a vessel containing 1 cc. of buffer solution. The vessel was then carefully attached to the respirometer which

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<sup>6</sup> This could not be determined absolutely but, by comparing dry weights of the residues from sterile agar and from agar plus fungus discs dropped into boiling water, it was found that the residues from sterile agar discs of comparable ages were heavier. The glucose and chloride content of the water used to separate the agar from mycelia was very small in comparison with analyses for the same substances in water in which sterile agar discs had been dissolved. However, Day (1942) in another connection, using a rather more drastic hot water extraction found mycelia to average 12–15% lower dry weights than comparable untreated mycelia. She melted agar off in hot water, fished out the mycelia (of *Phycomyces*) and immersed the mat in hot water before drying.



was placed in a water bath and allowed to equilibrate for 15 minutes before the initial reading was taken. Readings of oxygen consumption (as measured in 1/100 mm. decrease in the micrometer setting necessary to obtain levelling of the manometer) were made at 15 minute intervals during a period of one or more hours to determine the respiration rate (basal  $QO_2$ ) of the mycelia in a non-nutrient medium (buffer solution). At the end of this period, 0.5 cc. of 3x the desired final concentration of carbohydrate, poison, *etc.*, was introduced into the vessel and a second series of readings of oxygen consumption was made for a period of one or more hours. The time elapsing between the end of a control run and the beginning of an experimental run was usually 15 minutes. At the end of an experimental run a third run was undertaken in some cases, *e.g.*, return of the fungus to plain buffer after removal of a poison solution, or addition of a second compound in an attempt to counteract a poison.

Only preparations from liquid cultures were used in studies with added substrate, since the mycelia on agar discs already have nutrient available. No oxygen consumption was noted when controls of sterile agar discs were placed in the respirometer. No attempt was made to starve the mycelia obtained from liquid media by preliminary storage in plain buffer. The few experiments to be reported on the effect of various substrates on oxygen consumption were preliminary for the inhibition studies wherein only vigorous growths were to be employed. No difference in the  $QO_2$  was observed when a disc of mycelia on agar was placed in the respirometer vessel with agar or fungus side face down in this liquid; this was taken to mean the rate of oxygen diffusion into the liquid was not limiting. This was convenient since it was not desired to agitate the organisms in the vessels violently. The stream of air passed through the water in the bath created a slight vibration of the vessels through the flexible rubber tube mounting sufficient to agitate the liquid and organisms in the vessel; this procedure was deemed sufficient in view of the above finding reflecting on the low  $QO_2$  of these organisms.

*D. Description of Respirometer.* Since this is the first time the Scholander respirometer has been used with preparations of microorganisms, the following details of its operation are presented. The instrument and the vessels employed are shown in Fig. 1. The water bath in which the instrument is immersed was of glass and sufficiently deep to cover the thermobarometer (E) and oxygen reservoir (B) as well as the respiration vessel (C). A constant bubbling of air through the water in the bath is necessary to insure uniform mixing. Bubbling was accomplished easily by leading compressed air through a short length of 3 mm. glass tubing into a small disc of sponge rubber from which the air emerged as minute bubbles. The mercury reservoir must be free from air bubbles and the oxygen reservoir filled with water-saturated oxygen. The manometer is levelled against the edge of a white card held in place upon the composition backing by a clamp. The tubing connecting the respiration vessel needle to the instrument capillary should be of butadiene synthetic rather than natural rubber to avoid absorption of gas when exposed to pure oxygen. All rubber connections are securely wired to the glass tubing. Stopcocks are greased with a sulfur-free hydrocarbon grease (such as "Nevastane"). Micrometer readings are converted directly into mm.<sup>3</sup> through multiplication by a constant. Blank runs, identical with experimental runs but without organisms, produced only fractions of one mm.<sup>3</sup> changes over a one hour period.

## OXYGEN CONSUMPTION IN PRESENCE OF CARBON SOURCES

After a preliminary equilibration period of 15 minutes, the rate of oxygen consumption for all organisms examined was linear for several hours in the absence of added substrate. Such was true for preparations from either agar or liquid cultures. Evidently sufficient oxidizable

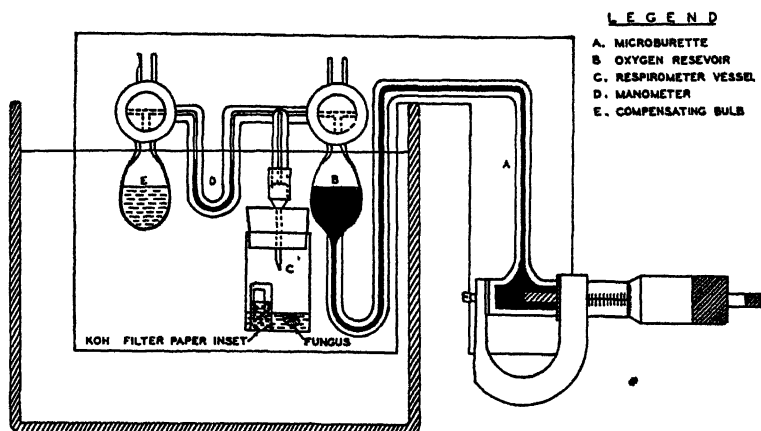


FIG. 1

## Scholander Volumetric Microrespirometer

Constant pressure obtained by turning ratchet of micrometer to level manometer. A film of water lies on the mercury in B; no free mercury surface is exposed; liquid in D is manometer fluid.

reserve stores are readily available to these fungi to permit a relatively constant basal (endogenous?) rate of oxygen consumption. This was the experience of Schade and Thimann (1940) with *Leptomitius lacteus* and of Wolf and Shoup (1943) with four species of *Allomyces*. Considering a change of  $\pm 0.10$  from the basal  $Q_{O_2}$  to be significant, no carbon source was found that promoted an increased oxygen consumption over the basal rate in  $M/15 \text{ KH}_2\text{PO}_4$  at pH 4.6; data are shown in Table I (by  $Q_{O_2}$  is meant  $\text{mm}^3 \text{ O}_2$  consumed/mg. dry weight/hr.). In Sorensen phosphate buffer at pH 8.0, the addition of  $M/20$  dextrose was followed by an immediate rise of 43% in the  $Q_{O_2}$  of *T. gypseum*, while addition of  $M/30$  glycerol to *T. rubrum* increased its  $Q_{O_2}$  by 36%. The rates following addition of substrate were constant for a period of one or more hours at these higher levels. All other

carbon sources tested for oxidizability were either without effect or toxic. In particular, the fatty acids were toxic and, surprisingly, *M/100* sucrose at both pH 4.6 and 8.0 was inhibitory for *T. rubrum*.

TABLE I

*Effect of Various Carbon Sources on Oxygen Uptake by Two Species of Dermatophytes*

Buffers employed were: pH 4.6 *M/15*  $\text{KH}_2\text{PO}_4$ , pH 7.0  $\text{CO}_2$ -free distilled water, pH 8.0 (in expt. No. 90) citrate-phosphate, pH 8.0 (in other expts.) *M/15*  $\text{Na}_2\text{HPO}_4$ -*M/15*  $\text{KH}_2\text{PO}_4$ . Basal  $\text{QO}_2$  determined in plain buffer for one or more hours immediately preceding addition of C-source; 28°C.; atmosphere  $\text{O}_2$ ; fungus preparations not starved.

Expt. no.	Carbon source	Concentration	Organism	pH	Basal $\text{QO}_2$	$\text{QO}_2$ carbon source	Per cent change in rate of $\text{O}_2$ uptake
8	Dextrose	<i>M/20</i>	<i>T. rubrum</i>	4.6	1.44	1.44	0
18	Dextrose	<i>M/20</i>	<i>T. rubrum</i>	4.6	1.28	1.27	0
90	Dextrose	<i>M/20</i>	<i>T. gypseum</i>	8.0	2.69	3.86	+43
153	Sucrose	<i>M/100</i>	<i>T. rubrum</i>	4.6	0.23	0.11	-52
157	Sucrose	<i>M/100</i>	<i>T. rubrum</i>	8.0	0.55	0.32	-42
150	Mannitol	<i>M/60</i>	<i>T. rubrum</i>	4.6	0.23	0.21	0
135	Mannitol	<i>M/60</i>	<i>T. rubrum</i>	8.0	0.55	0.55	0
151	Glycerol	<i>M/30</i>	<i>T. rubrum</i>	4.6	0.23	0.21	0
156	Glycerol	<i>M/30</i>	<i>T. rubrum</i>	8.0	0.55	0.75	+36
164	Soluble starch	1/6%	<i>T. rubrum</i>	8.0	0.87	0.92	0
165	Calcium gluconate	<i>M/120</i>	<i>T. rubrum</i>	8.0	0.87	0.79	0
170	Na formate	<i>M/20</i>	<i>T. gypseum</i>	4.6	0.64	0.57	-10
73	Na citrate	<i>M/100</i>	<i>T. gypseum</i>	4.6	1.06	0.92	-13
229	Bactotryptose	1/3%	<i>T. gypseum</i>	7.0	1.73	1.56	-10
230	Asparagine	<i>M/40</i>	<i>T. gypseum</i>	7.0	1.73	1.74	0

It seems clear that the basal rate of oxygen consumption with these organisms is not comparable to the endogenous rate (rate of  $\text{O}_2$  uptake in the absence of added substrate) with yeasts. It is not likely that we are here dealing with surface saturation of enzymes by substrate or questions of permeability. Possibly these organisms are equipped only to oxidize some assimilation product, possibly carbohydrate, to which the dextrose and glycerol might have been converted at pH 8.0.

TABLE II

*Respiration of T. rubrum at Different Hydrogen Ion Concentrations  
Using Various Buffers*

Buffer composition as indicated; 28°C.; atmosphere, O<sub>2</sub>

Expt. no.	Buffer	pH	Dry weight	Duration of expt.	Total O <sub>2</sub> consumed	Q <sub>O<sub>2</sub></sub>
			mg.	hrs.	mm. <sup>3</sup>	
167	M/15 KH <sub>2</sub> PO <sub>4</sub>	4.6	60.1	1½	69.7	0.93
168	KHPhthalate + NaOH	4.0		1½	66.7	0.89
169	KHPhthalate + HCl	3.0		1½	58.6	0.78
170	KCl + HCl	2.0		1½	28.8	0.38
171	M/15 KH <sub>2</sub> PO <sub>4</sub>	4.6		1½	28.0	0.37
173	KHPhthalate + NaOH	4.0	40.3	1½	49.4	0.98
174	KHPhthalate + HCl	3.0		1½	45.5	0.90
175	KCl + HCl	2.0		1½	29.8	0.59
176	M/15 KH <sub>2</sub> PO <sub>4</sub>	4.6		1½	31.6	0.63
177	KH <sub>2</sub> PO <sub>4</sub> + NaOH	8.0	54.7	1½	52.7	0.77
178	KH <sub>2</sub> PO <sub>4</sub> + NaOH	7.0		1½	51.2	0.75
179	KHPhthalate + NaOH	6.0		2½	94.2	0.69
180	M/15 KH <sub>2</sub> PO <sub>4</sub>	4.6		1½	52.2	0.64
181	KH <sub>2</sub> PO <sub>4</sub> + NaOH	8.0	62.8	1½	72.4	0.92
182	KH <sub>2</sub> PO <sub>4</sub> + NaOH	7.0		1½	57.2	0.73
183	KHPhthalate + NaOH	6.0		2½	118.6	0.76
184	M/15 KH <sub>2</sub> PO <sub>4</sub>	4.6		1½	62.2	0.66
185	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	8.0	75.0	1½	70.8	0.75
187	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	6.0		1½	71.5	0.76
189	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	4.0		2½	106.5	0.57
190	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	8.0	59.7	1½	45.5	0.61
191	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	7.0		1½	43.9	0.59
192	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	6.0		1½	46.1	0.62
193	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	5.0		1½	38.2	0.51
194	Citrate—Na <sub>2</sub> HPO <sub>4</sub>	4.0		2½	57.6	0.39

#### EFFECT OF HYDROGEN ION CONCENTRATION ON OXYGEN CONSUMPTION

As shown in Fig. 2, there is a depression in the Q<sub>O<sub>2</sub></sub> — pH curve in the region of pH 5.0–5.5 for *E. floccosum* and *T. gypseum*. Ordinarily one finds an optimum pH range for oxygen consumption with most

organisms. The finding of a depression in the  $Q_{O_2}$  at a pH range flanked by an increase in rate at both higher and lower H-ion concentrations seems to have been reported only once before, by Tang (1936). The rate of oxygen consumption at any given pH was constant for the period the organism was held there, always one or more hours. The pH of the buffers was checked with a glass electrode after being made, and again, colorimetrically, immediately before use.

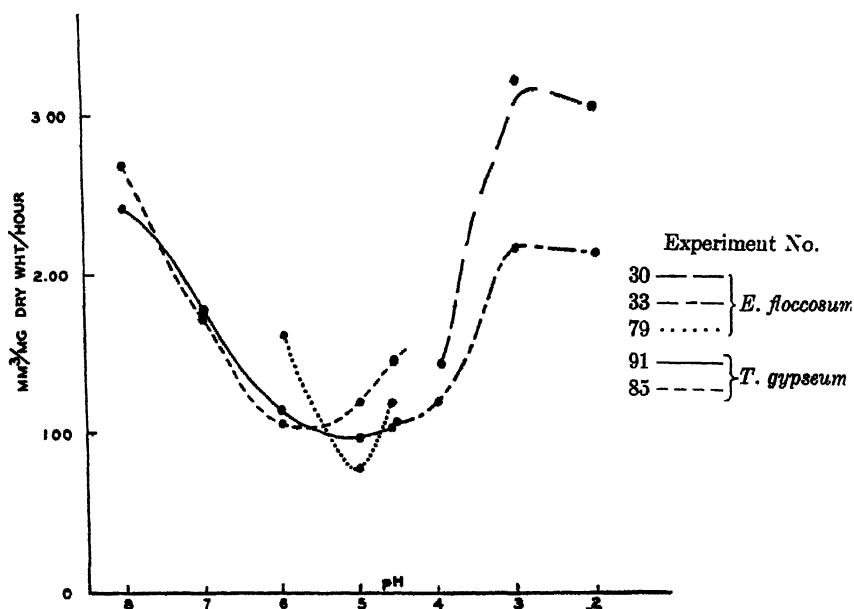


FIG. 2

Variation in the  $Q_{O_2}$  of *E. floccosum* and *T. gypsum* with pH

Each point is the rate for  $O_2$  uptake over a period of 1 or more hours; Mollvaine's citrate-phosphate buffer used. Dry weights of fungus used were: Expt. No. 30, 10.0 mg.; No. 33, 9.9 mg.; No. 79, 17.7 mg.; No. 85, 77.4 mg.; No. 91, 64.2 mg. Temperature 28°C.; atmosphere,  $O_2$ .

Employing *T. rubrum* and phthalate, citrate and phosphate series of hydrogen ion buffers we found that the rate of oxygen consumption decreased slightly as the H-ion concentration increased; this variation in  $Q_{O_2}$  with pH was independent of the buffer employed. *T. rubrum* was shown (Nickerson, 1946) to have, on the average, a basal  $Q_{O_2}$  at

constant pH markedly lower than found for the other dermatophytes. There seems to be no tendency for the  $Q_{O_2}$  of *T. rubrum* to be particularly affected by the H-ion concentration of its environment.

In discussing the localization of enzymes within cells, Heilbrunn (1943) points to the work of Myrbäck and Vasseur (1943) on lactose-fermenting yeasts who have claimed that enzymes localized at the cell surface are markedly affected by the pH of the cell environment while enzymes located within the cell are not easily so influenced. Tang (1936), studying the rate of oxygen consumption by *Saccharomyces wanching* at different H-ion concentrations in different buffer systems, found the chemical nature of the buffer system anions to be of considerable importance. It is well known that protoplasm is affected differently by different acids; the citrate ion (employed in the buffer for Fig. 2) is usually placed first in the list of anions in a lyotropic series of substances causing reversible protein precipitation (see Gortner, 1938).

#### EFFECT OF SALT CONCENTRATION ON OXYGEN CONSUMPTION

Different concentrations of sodium chloride were added to respiring preparations of *T. gypsum* in distilled water. It was found that the  $Q_{O_2}$  was highest in distilled water, falling sharply with the addition of small amounts of NaCl only to increase with slightly greater concentrations of salt. As in the pH studies, the fungus was held at each salt concentration level for a period of one or more hours, during which time the rate was constant. This behaviour with NaCl is interesting, particularly in view of the reaction of *E. floccosum* and *T. gypsum* to changes in pH. The response of *T. gypsum* to changes in salt concentration (Fig. 3) is reminiscent of the variation in solubility of a globulin with change in salt concentration (see Gortner 1938).

Analogies may be drawn between the behavior of proteins and the respiration of *E. floccosum* and *T. gypsum* with changes in pH and salt concentration in the cell environment. While these analogies are interesting, we feel they can only serve at the moment to indicate future lines of work. The enzymes controlling respiration in some species of dermatophytes may lie in or near the surface easily influenced by environmental conditions, and possess components with an isoelectric point at pH 5.0-5.5, and globulin in nature.

### EFFECT OF CERTAIN INORGANIC COMPOUNDS ON OXYGEN CONSUMPTION

The selection of substances to be studied for their effect on oxygen consumption was directed mainly toward (a) inorganic water-soluble salts and (b) salts of fatty acids. Attention to (a) was drawn by observations of Salvin (unpublished) on the toxic action of zinc

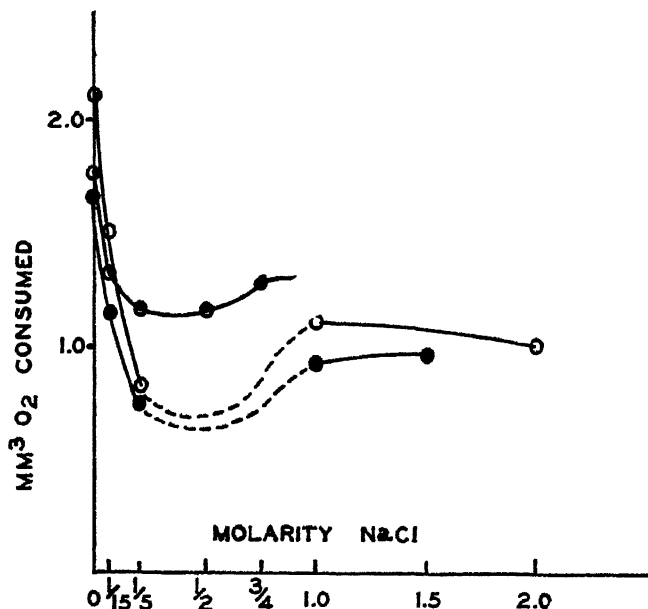


FIG. 3

Oxygen Consumption by *T. gypseum* at Different Concentrations of Sodium Chloride

Zero concentration (basal rate) in CO<sub>2</sub>-free distilled water. Organisms held 1¼ hours at each concentration; temperature, 28°C.; atmosphere, O<sub>2</sub>. Open circles, expt. nos. 220-224, 226 mg. dry wt.; closed circles, expt. nos. 213-217, 28.4 mg. dry wt.; half closed circles, expt. nos. 231-235, 29.9 mg. dry wt.

compounds toward *Aspergillus niger* as shown by manometric measurements on respiration; by similar observations of Nickerson (unpublished) on the inhibitory effect of zinc chloride on *Trichophyton Schoenleinii*; and by the work of McCallan and Wilcoxon (1934).

Results of determinations of oxygen consumption in the presence of salts of certain inorganic compounds are listed in Table III. Respiration

TABLE III

*Effect of Inorganic Salts, other than Zinc, on O<sub>2</sub> Uptake by E. floccosum and T. rubrum*

Rate in  $M/15 \text{ KII}_2\text{PO}_4$  is the basal rate. Expt. no. indicates order of treatment. 28°C.; atmosphere, O<sub>2</sub>.

Expt. no.	Organism	Treatment	Qo <sub>2</sub>	Change from basal Qo <sub>2</sub>
				per cent
23	<i>E. floccosum</i>	$M/15 \text{ KII}_2\text{PO}_4$	2.45	
24		$M/100 \text{ RbCl}$	2.56	0
101	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	1.08	
105		$M/100 \text{ AgNO}_3$		-100
106		$M/15 \text{ KII}_2\text{PO}_4$		no recovery
108	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	1.04	
109		$M/10,000 \text{ AgNO}_3$	0.59	-43
143	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	0.48	
145		$M/100 \text{ CdSO}_4$	0.66	+37
146	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	0.80	
148		$M/100 \text{ CdCl}_2$	0.62	-22
195	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	0.43	
196		$M/1000 \text{ HgCl}_2$	0.31	-28
197		$M/100 \text{ HgCl}_2$	0.11	-74
198	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	0.50	
199		$M/1000 \text{ HgCl}_2$	0.36	-28
200		$M/100 \text{ HgCl}_2$	0.08	-84
7	<i>T. rubrum</i>	$M/15 \text{ KH}_2\text{PO}_4$	1.44	
8		$M/20 \text{ Dextrose}$	1.44	0
9		$M/100 \text{ NaF}$	1.62	+12

of the fungi in the presence of various inorganic zinc compounds is shown in Table IV. As mentioned under previous headings, the rate of oxygen uptake was constant for the test period during which the fungus was exposed to treatment. It is clear that some zinc compounds are about as effective as salts of silver or mercury for inhibiting respiration. Zinc chloride retains some activity even in dilutions of  $10^{-4} M$  against all organisms tested. The effect of  $10^{-2} M$  zinc chloride



persists even after the fungus had been removed from the presence of the salt and well washed with phosphate buffer or distilled water (Fig. 4).

TABLE IV

*Effect of Inorganic Zinc Salts on O<sub>2</sub> Consumption by Certain of the Dermatophytes*

Rate in *M/15 KH<sub>2</sub>PO<sub>4</sub>* is the basal rate, except for expt. no. 37.; expt. no. indicates order of treatment; 28°C.; atmosphere, O<sub>2</sub>.

Expt. no.	Organism	Treatment	Q <sub>O<sub>2</sub></sub>	Change from basal Q <sub>O<sub>2</sub></sub>
				<i>per cent</i>
116	<i>T. rubrum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	1.02	
117		<i>M/1000 Zn acetate</i>	1.30	+27
137	<i>T. rubrum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	1.40	
138		<i>M/1000 Zn(NO<sub>3</sub>)<sub>2</sub></i>	1.09	-22
25	<i>E. floccosum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	1.61	
26		<i>M/100 ZnCl<sub>2</sub></i>	0.65	-60
37	<i>E. floccosum</i>	CO <sub>2</sub> -free H <sub>2</sub> O	2.36	
38		<i>M/100 ZnCl<sub>2</sub></i>	0.20	-92
39		CO <sub>2</sub> -free H <sub>2</sub> O	0.84	-65
40	<i>E. floccosum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	3.23	
41		<i>M/100 ZnCl<sub>2</sub></i>	0.24	-93
42		<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	1.32	-59
63	<i>T. gypsum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	2.34	
64		<i>M/10,000 ZnCl<sub>2</sub></i>	2.04	-13
66	<i>T. gypsum</i>	<i>M/15 KH<sub>2</sub>PO<sub>4</sub></i>	2.85	
67		<i>M/1000 ZnCl<sub>2</sub></i>	2.18	-24

A reversal of the toxic effects of zinc salts following the addition of calcium chloride has been reported for animals (Sutton, 1939; Jones, 1938) and for bacteria following the addition of sodium thiosulfate (Vignati and Schnabel, 1928). No evidence for such a phenomenon was found with the fungi employed here as judged by the rates of oxygen uptake. The addition of *M/1000 CaCl<sub>2</sub>* following *M/1000 Zn(NO<sub>3</sub>)<sub>2</sub>* with *T. rubrum* only served to depress oxygen uptake slightly more; the experience with sodium thiosulfate and magnesium

sulfate following other zinc salts was similar (Table V). In the other direction, attempts to augment the action of zinc salts by preliminary use of detergents were not notably successful.

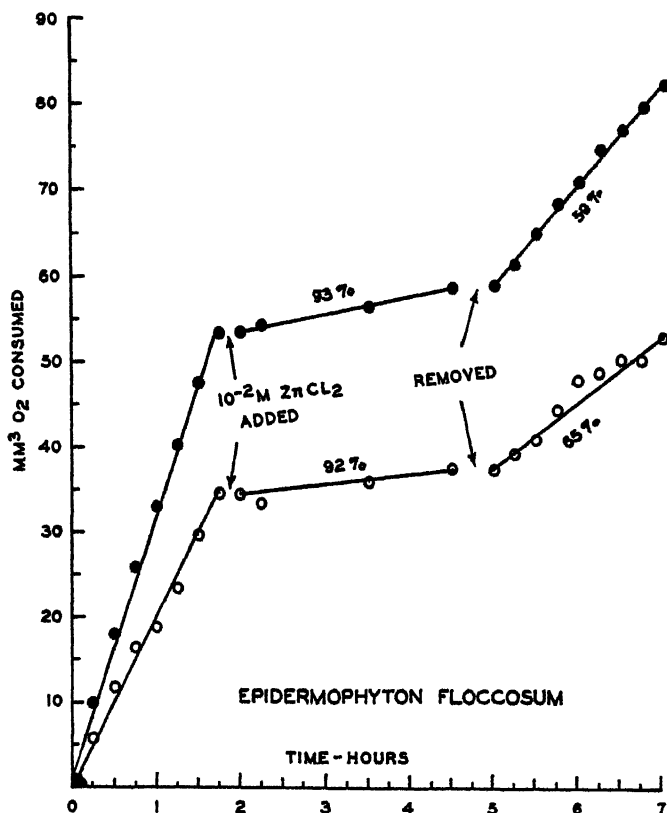


FIG. 4

Effect of Addition of  $M/100$  Zinc Chloride upon the Rate of  $O_2$  Uptake by *E. floccosum*

For experiment starting in  $M/15 K_2H_2PO_4$ , 9.0 mg. dry weight (closed circles); for expt. in  $CO_2$ -free distilled water, 8.9 mg. dry weight (open circles);  $28^\circ C$ . Values indicated are *per cent* decrease in consumption as compared with the respective basal rates. Fungus preparations returned to buffer or water at 5 hour mark.

#### EFFECT OF CERTAIN ORGANIC COMPOUNDS ON RESPIRATION

In the fatty acid series (Table VI) addition of  $M/100$  propionate to *E. floccosum* resulted in three cases in inhibitions of 35, 27 and 13%

respectively. This concentration was evidently not fungicidal since return of the fungus to phosphate buffer was followed by an immediate return of the  $Q_{O_2}$  to its initial basal level in buffer. Sodium undecylenate<sup>7</sup> in  $M/200$  concentration promoted a 37% decrease in the rate of respiration of *T. rubrum*.

TABLE V

*Attempts to Overcome Zinc Inhibition by Addition of Other Compounds*

Rate in  $M/15$   $KH_2PO_4$  is basal rate; 28°C.; atmosphere,  $O_2$ ; zinc compound removed and fungus washed with three changes of second compound within 15 minute period.

Expt. no.	Organism	Treatment	$Q_{O_2}$	Change from basal $Q_{O_2}$
				<i>per cent</i>
63	<i>T. gypseum</i>	$M/15$ $KH_2PO_4$	2.34	
64		$M/10,000$ $ZnCl_2$	2.04	-13
65		$M/1000$ $MgSO_4$	2.14	0
66	<i>T. gypseum</i>	$M/15$ $KH_2PO_4$	2.85	
67		$M/1000$ $ZnCl_2$	2.18	-23
68		$M/100$ $MgSO_4$	1.37	-52
137	<i>T. rubrum</i>	$M/15$ $KH_2PO_4$	1.40	
138		$M/1000$ $Zn(NO_3)_2$	1.09	-22
139		$M/1000$ $CaCl_2$	0.77	-45
201	<i>T. rubrum</i>	$M/15$ $KH_2PO_4$	0.45	
202		$M/1000$ $ZnCl_2$	0.36	-20
203		$M/1000$ $Na_2S_2O_3$	0.26	-42

Among other organic compounds tested for inhibitory effect on respiration,  $4.5 \times 10^{-4}$   $M$  phenyl mercuric nitrate caused a 40% drop in the  $Q_{O_2}$  of *T. gypseum* (Table VII). This concentration was transitory in effect since return of the fungus to plain buffer was followed by a noticeable rise in the  $Q_{O_2}$  from its depressed level. It will be noted that the inhibition following addition of phenyl mercuric nitrate was greater than that resulting from a stronger concentration of mercuric chloride ( $M/1000$ ). Addition of  $1.5 \times 10^{-3}$   $M$  hexylchloro *m*-cresol<sup>7</sup> to

<sup>7</sup> We are indebted to Dr. H. W. Hartman, Henry Ford Hospital, Detroit for a sample of hexylchloro *m*-cresol, and to Dr. L. Reiner, Wallace and Tiernan Company, for a sample of undecylenic acid.

*T. gypsum* in the presence of Na formate was followed by a decline of 44% of the rate in buffer. With the diphenyls, curiously, *o*-diphenyl was stimulatory while the *p*- form was slightly inhibitory.

TABLE VI

*Effects of Some Organic Acids on O<sub>2</sub> Uptake by T. rubrum and E. floccosum*

Rate in M/15 KH<sub>2</sub>PO<sub>4</sub> is basal rate; salts of acids made up in this buffer and used at pH 4.6; 28°C.; atmosphere, O<sub>2</sub>.

Expt. no.	Organism	Treatment	Q <sub>O<sub>2</sub></sub>	Change from basal Q <sub>O<sub>2</sub></sub>
				per cent
4	<i>T. rubrum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	1.22	
5		M/20 Dextrose	1.02	-15
6		M/100 Na malonate	0.96	-20
46	<i>E. floccosum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	1.10	
47		M/100 Na propionate	0.70	-35
48		M/15 KH <sub>2</sub> PO <sub>4</sub>	1.71	+55
49	<i>E. floccosum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	1.66	
50		M/100 Na propionate	1.20	-27
51		M/15 KH <sub>2</sub> PO <sub>4</sub>	1.54	0
129	<i>T. rubrum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	0.81	
130		M/200 Na undecylenate	0.51	-37
112	<i>T. rubrum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	1.19	
113		M/1000 H <sub>3</sub> BO <sub>3</sub>	0.58	-51
82	<i>E. floccosum</i>	M/15 KH <sub>2</sub> PO <sub>4</sub>	2.11	
83		M/100 Na propionate	1.83	-13
84		M/15 KH <sub>2</sub> PO <sub>4</sub>	2.37	+12

## DISCUSSION

In a borate buffer series (KCl-NaOH-Boric acid) using *Saccharomyces wanching*, Tang (1936) found a minimum rate of oxygen consumption at about pH 8.9, "The general shape of the curve for this pH range resembles that characteristic of many colloidal processes and suggests that there exists in the cells of *S. wanching* a mechanism of respiration having some specifically colloidal factor with an isoelectric point at pH 8.9." (We were unable to use a borate buffer

TABLE VII

*Effect of Certain Organic Compounds on Oxygen Consumption by T. gypsum*

Rate in  $M/15 \text{ KH}_2\text{PO}_4$  is the basal rate;  $28^\circ\text{C}.$ ; atmosphere,  $\text{O}_2$ ; the diphenyls were made up as saturated solutions and 0.5 cc. added to 1 cc. phosphate buffer in the vessels; final concentration is quoted as a  $1/3$  saturated solution.

Expt. no	Treatment	$\text{QO}_2$	Change from basal $\text{QO}_2$
			<i>per cent</i>
57	$M/15 \text{ KH}_2\text{PO}_4$	0.58	
58	$4.5 \times 10^{-4} M$ Phenyl mercuric nitrate	0.35	-40
59	$M/15 \text{ KH}_2\text{PO}_4$	0.44	-24
69	$M/15 \text{ KH}_2\text{PO}_4$	0.64	
70	$M/20 \text{ Na formate}$	0.57	-10
71	$1.5 \times 10^{-3} M$ Hexylchloro <i>m</i> -cresol	0.36	-44
75	$M/15 \text{ KH}_2\text{PO}_4$	2.45	
76	$1/3 \text{ sat. sol'n. } p\text{-diphenyl}$	2.04	-17
77	$M/15 \text{ KH}_2\text{PO}_4$	1.94	
78	$1/3 \text{ sat. sol'n. } o\text{-diphenyl}$	2.35	+21

because of the inhibitory effect of boric acid on these organisms, see Table VI). In contrast to the behaviour of the yeast in borate buffer, its rate of oxygen consumption in phthalate and phosphate buffers was found to decline gradually towards the extreme acid or alkaline ranges; with an acetate buffer the rate dropped precipitously with increasing acidity. Tang believed he could distinguish two respiratory systems in his yeast, one higher in rate and lowered by increasing acidity; the other, having a component with an isoelectric point at pH 8.9 and lower in rate. With the organisms studied here it appears that the respiratory system of *T. rubrum* may be different from that possessed by the other organisms examined. *T. rubrum* has a lower, less variable basal rate of oxygen consumption and is not markedly affected by the pH of its environment. The evidence, however, permits little choice between thinking of the respiratory system of *T. rubrum* possessing different components or having comparable components located within the cell interior. These observed differences of behaviour for *T. rubrum* are of considerable interest when one recalls that it is the most stubborn of the causative organisms for tinea pedis, being

extremely difficult to remove as Conant *et al.* (1945) point out; the connection, if any exist, between the differences observed in the laboratory for *T. rubrum* and the clinical findings is to be explained.

Borei and Lindvall (1943) showed the endogenous respiration of *Saccharomyces cerevisiae*, proceeding according to the cytochrome oxidase-cytochrome system, consists of two fractions. With increasing acidity (pH 6.5 to 4), one fraction is unchanged while the other increases in rate, probably as a result of enzyme activation.

In connection with the findings of Tang and of the present paper, the recent caution of van Goor and Jongbloed (1943) should be pointed out. These authors, attempting to repeat work of Overbeek and Polder (1941) on oxygen consumption by minced animal tissue, obtained a curve for oxygen uptake with time exhibiting a minimum flanked by higher rates (see Fig. 2 of this paper). van Goor and Jongbloed showed the upswing in rate after several hours to be a result of bacterial contamination and not of glycogen synthesis, as Overbeek and Polder had claimed. In the present instance it is believed contamination does not enter the picture, since considerable care was exercised to maintain as nearly aseptic conditions as possible; furthermore, the lack of substrate and high acidity in most experiments were not conducive to bacterial growth.

The preponderant role of assimilative processes with these organisms is emphasized by our findings that, under acid conditions, no added carbon source was oxidized and at alkaline levels only two substances promoted an increase in oxygen uptake over control values. Findings of Robbins and Ma (*loc. cit.*) on assimilative processes in nitrogen nutrition were mentioned previously. Indeed, one might not be surprised that an essentially low-grade parasite dwelling in the comparatively impoverished epidermis has survived through powers of assimilation when dissimilatory mechanisms would find substrates scanty.

A great deal has been written about the biological action of zinc and of zinc salts; an interesting review by Hegsted, McKibbin and Drinker (1945) summarizes a large amount of work on the biological importance of this element. From our results, zinc salts seem nearly as toxic as salts of mercury and silver to certain species of dermatophytes and much more toxic than salts of cadmium, in contrast to the findings of Gutstein (1932). Gutstein believed that heavy metal compounds act by precipitating phosphatides in the cell walls. However, the lack of

sequence in the activity of compounds, within a class in the periodic table, with increasing atomic weight would seem to argue against any single mode of action for the metals within that class.

Inhibition of fungus respiration as a metabolic bioassay method has been proposed (Nickerson, 1946) as a means for obtaining relevant laboratory data on the effectiveness of chemical agents in controlling undesirable fungus growths under conditions more closely approximating operational (*i.e.*, effect on vigorous, metabolizing mycelia) than hitherto employed. Part of the basis for this proposal has been detailed in the present paper. It is believed that these procedures may profitably be applied to gain information on the action of fungicides and to supply criteria for the selection and performance of fungicides. It is believed that a method (to be used in addition to the existing growth-test and spore germination-test procedures) supplying information on the effect of chemicals on fungi in a mycelial state may have application in laboratory studies on the fungus deterioration of equipment and on pathogenic fungi.

It is a pleasure to acknowledge the encouragement of Lt. Col. Laurence Irving and Lt. Col. J. R. Scholtz in this work, and the discussions with Major P. F. Scholander and Lt. G. A. Edwards; technical assistance from Stuart Lee is appreciated. The authors are indebted to Dr. K. V. Thimann for reading the manuscript.

### SUMMARY

Methods are described for handling mycelial organizations of filamentous fungi for study of oxygen consumption in respirometers. The use of studies on the inhibition of respiration as a means of evaluating the action of chemicals *in vitro* is recommended as a promising adjunct to methods at present in use.

Oxygen consumption by three species of dermatophytes: *Trichophyton rubrum*, *T. gypsum* (mentagrophytes) and *Epidermophyton floccosum* has been examined. No increase in oxygen consumption was found at pH 4.6 for any organism with any of several carbon sources tested. At pH 8.0, *M/20* dextrose produced a significant increase in the  $Q_{O_2}$  when added to respiring preparations of *T. gypsum*; *M/30* glycerol did likewise for *T. rubrum*.

With a citrate buffer series the rate of oxygen consumption by *E. floccosum* and *T. gypsum* reached a minimum at pH 5.0–5.5, with higher rates at more acid or alkaline levels. The  $Q_{O_2}$  of *T. gypsum* fell rapidly with the addition of low concentrations of sodium chloride

to mycelia respiring in distilled water;  $O_2$  uptake increased, however, with the addition of more NaCl. Analogies are drawn between the behavior of proteins and the respiratory systems of these two organisms. The respiratory systems may be in or near the cell surface readily affected by environmental changes, possess components with an isoelectric point at pH 5.0–5.5, and be of a globulin nature.

$O_2$  uptake by *T. rubrum* showed only a slight decline with increasing acidity; this relationship was independent of the buffer system employed. The mean basal  $Q_{O_2}$  for 26 isolates of *T. rubrum* was markedly less than for the other species. These and other differences found in our study separating *T. rubrum* from two other dermatophytes are interesting since *T. rubrum* differs clinically from other species; greater difficulty is usually experienced in curing infections in which *T. rubrum* is the causative organism.

The effect of certain water-soluble inorganic salts on  $O_2$  uptake was studied; salts of zinc, mercury and silver were found to be highly inhibitory, while cadmium compounds had little effect. The inhibition produced by zinc salts could not be overcome by the addition of calcium chloride, sodium thiosulfate or magnesium sulfate.

Salts of fatty acids were found to depress  $O_2$  consumption to varying extents;  $M/100$  sodium propionate was slightly inhibitory, but  $O_2$  uptake rose to the basal level after removal of the salt from the environment.

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# A Submicro Method for the Determination of Iodine Number of Lipids<sup>1</sup>

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## INTRODUCTION

It is frequently desirable to know the degree of unsaturation of very small samples of lipid. The existing micro modifications of iodine number determinations require larger amounts of lipid than can be obtained from 0.1 ml. blood samples. The present study was made to devise a micro method requiring 10–100  $\gamma$  of lipid, depending on the degree of unsaturation.

The determination of iodine number has been used for over half a century in lipid analysis and a variety of halogenating reagents have been used to add to the double bonds of the unsaturated fatty acids. Rosenmund and Kuhnhehn in 1923 (4) were the first to apply the pyridine sulfate dibromide reagent in the determination of iodine number. Yasuda (6) modified this method so that samples as small as 3–5 mg. could be used and this procedure has been applied in many biological studies. Wilson and Hansen (5), utilizing the Yasuda method for the determination of lipid unsaturation of plasma or serum, added hydroquinone as an antioxidant during lipid extraction. The hydroquinone remains in the aqueous phase when the lipid is re-extracted with petroleum ether and, therefore, does not interfere with the determination.

The present adaptation of the Rosenmund-Kuhnhehn method to submicro quantities of fat consists largely in the design and construction of suitable equipment.

## EXPERIMENTAL

To deliver small samples (.05 ml.) of pyridine sulfate dibromide a pipette was constructed as described by Kirk (1).

<sup>1</sup> The experimental data are taken from a thesis submitted to the Graduate School of the University of Minnesota by Norman Kretchmer in partial fulfillment of the requirements for the degree of Master of Science.

The burette used was a modification of the Rehberg burette (3) having a bore such that one centimeter length equals 2.72 cu. mm. and having a total capacity of 197.2 cu. mm. Inasmuch as mercury does not react with thiosulfate the burette designed for this reagent contains a direct contact mercury drive. The titration is carried out with the tip of the burette beneath the surface of the reaction mixture; and constant stirring is effected by a glass-covered iron pellet similar to the "flea" described by Linderstrøm-Lang (2), which is moved up and down by an intermittent magnetic field giving 4 pulses per second. The alternations are produced by connections across the commutator of a small motor (Fig. 1).

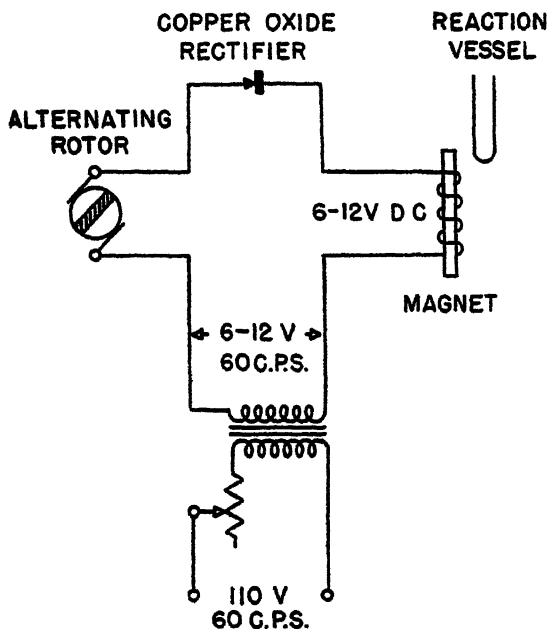


FIG. 1  
Wiring Diagram of the Magnetic Mixer

Reagents:

- (a) Redistilled chloroform (alcohol-free)
- (b) 0.02 *N* sodium thiosulfate (in water)
- (c) 0.05 *N* pyridine sulfate dibromide (in glacial acetic acid)
- (d) 10% potassium iodide (in water)
- (e) 1% starch solution

The experimental procedure is a modification of that of Yasuda.

One-tenth ml. of chloroform containing the lipid is added to the reaction vessel, which is a 1 × 4 cm. test tube. The amount of lipid contained in the chloroform should

vary with the degree of unsaturation and should be of such magnitude that the sample titre is about three-fourths that of the blank. To this solution and a blank 0.05 ml. of pyridine sulfate dibromide reagent is added, the two solutions are mixed and placed in the dark for twenty minutes (Fig. 2), after which the vessels are removed and 0.05 ml. of 10% potassium iodide is added to each.

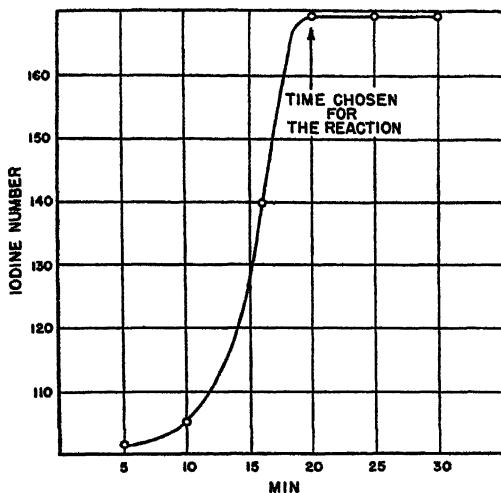


FIG. 2

Relation of Iodine Number to Time of Reaction

Three drops of 1% starch are then added to the mixture and the solution is titrated with 0.02 *N* sodium thiosulfate until colorless. The water delivered in the three drops of starch is sufficient to promote ionic reactions. It was found best to run a blank for each sample. However, if this is not done, it is imperative that at least two blanks be run with each group of samples. Acetic acid has a large coefficient of expansion and any considerable change in temperature brings about an error in the amount of pyridine sulfate dibromide delivered.

To minimize oxidation of the lipid solutions, they were preserved in the cold and in an atmosphere of carbon dioxide when not being used.

## RESULTS

The submicro modification was applied to a group of oils and a group of esters of pure fatty acids. The values obtained on the pure fatty esters are in close agreement with those given by the conventional macro Wijs method (Table I). The very small degree of variation shown

TABLE I

*The Submicro Modification of the Rosenmund-Kuhnhehn Iodine Number  
Compared with the Macro Wijs Iodine Number*

Lipid	Sample weight ( $\gamma$ )	Mean iodine value	Macro Wijs	Theoretical iodine value
Olive oil	89.1	82.9 $\pm$ 0.317 (8) <sup>2</sup>	83.2	-
Corn oil	62.3	124.8 $\pm$ 0.166 (8)	126.7	-
Linseed oil	45.9	169.8 $\pm$ 0.840 (10)	178.9	-
Ethyl oleate	53.5	79.8 $\pm$ (5)	80.4	79.8
	81.3	79.1 $\pm$ 0.250 <sup>1</sup> (3)		
Ethyl linoleate	40.9	160.5 $\pm$ (7)	160.6	163.6
	40.5	162.1 $\pm$ (4)		
	15.1	161.7 $\pm$ (3)		
	36.8	160.2 $\pm$ 0.290 <sup>1</sup> (2)		
Ethyl linolenate	18.0	246.0 $\pm$ 0.000 <sup>1</sup> (7)	243.7	247.0
Methyl arachidonate	20.4	309.6 $\pm$ 0.000 <sup>1</sup> (2)	316.5	317.3
	12.8	306.1 $\pm$ 0.680 <sup>1</sup> (4)		

<sup>1</sup> Figures represent standard error for all determinations made with each fatty acid ester.

<sup>2</sup> Numbers in parentheses indicate the number of determinations made on that sample.

for a given sample is due to the fact that these titrations were made on aliquot parts of the same solution. Hence, this was actually a measure of the accuracy of the pipets and buret as well as the reproducibility of the end point. The submicro modification was also applied to a few samples of whole blood and plasma. The iodine numbers obtained fell within the range established by Wilson *et al.* (5).

With the materials used in this study, where 1 cm. length of the burette is equal to 2.72 cu. mm., the calculation for iodine number becomes

$$\text{Iodine Number} = \frac{(\text{cm. blank} - \text{cm. sample})(.00272)}{\text{Weight of sample in g.}} \times \frac{1}{(\text{normality of thiosulfate}) (12.7)}$$

### SUMMARY

(1) A submicro modification of the Rosenmund-Kuhnhehn method for determination of the iodine value of lipids is described.

(2) The iodine value is determined on samples of 10–100  $\gamma$  in size.

(3) The method was applied to oils, fatty acids, whole blood (total lipids) and plasma fatty acids and gave iodine values which were in agreement with values obtained by the Wijs method.

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# The Folic Acid Content of Meats and the Retention of this Vitamin During Cooking\*

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## INTRODUCTION

Previous reports (1-8) from this laboratory have included values for the amount of each of seven different B vitamins in meat and for the retention of six of these vitamins during different cooking procedures. Although the significance of folic acid in animal nutrition is not completely known, sufficient information is available to indicate that folic acid and related compounds are active in several different species, including man (9-18). We have, therefore, continued our studies to include folic acid and the results for this vitamin are reported in the present paper.

## EXPERIMENTAL

The samples of meat used for the estimation of folic acid were obtained from local meat markets, ground in an electric meat grinder and mixed thoroughly. Portions were placed immediately in dark-colored bottles and stored in a  $-4^{\circ}\text{C}$ . cold room. A few of the samples were analyzed immediately and reanalyzed at intervals of a few weeks to determine whether any loss of this vitamin occurred during storage. Since the values did not decrease no attempt was made to analyze all the samples immediately. The preparation of the samples of meat used and the methods of cooking and curing were described in earlier reports (1, 4, 5).

The folic acid determinations were made by the method of Tepy and Elvehjem (19) using both *Streptococcus faecalis* and *Lactobacillus casei* as test organisms. Crystalline vitamin B<sub>12</sub> has been used as the folic acid standard throughout this work.

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<sup>1</sup> Kindly supplied by Parke, Davis and Company.



TABLE I  
Folic Acid Content of Meats

Sample	B <sub>9</sub> Potency		Sample	B <sub>9</sub> Potency	
	<i>S. faecalis</i>	<i>L. casei</i>		<i>S. faecalis</i>	<i>L. casei</i>
	<i>mg./100 g.</i>			<i>mg./100 g.</i>	
<i>Veal</i>			<i>Beef (cont.)</i>		
leg	.025	.018	chuck	.016	.011
leg	.029	—	chuck	.015	.010
leg	.033	.018	brain	.013	.012
leg	.021	.013	brain	.011	.012
sirloin chop	.031	.022	liver	.060	.086
sirloin chop	.014	.013	liver	.150	.105
shoulder	.026	.017	liver	.044	.056
shoulder	.026	.018	liver	.064	—
shoulder	.016	.012	kidney	.030	.035
shoulder	.015	.014	kidney	.043	.056
flank	.021	.016	<i>Lamb</i>		
liver	.040	.051	leg	.012	.011
liver	.052	.051	leg	.0082	.0064
			shoulder	.0082	.0068
liver	.039	.048	<i>Pork</i>		
liver	.039	.048	ham	.009	.0078
kidney	.031	.045	ham	.012	.0138
kidney	.036	.047	ham	.0135	.0075
heart	.013	—	ham	.0067	.0056
heart	.012	—	ham	.0088	—
			loin	.0069	—
<i>Beef</i>			liver	.063	—
round	.033	—	liver	.084	—
round	.021	—			
round	.024	—			
rib	.017	.011			

Several methods of hydrolyzing the tissues were used for the liberation of folic acid and it was found that incubation of the samples with takadiastase for 24 hours at pH 4.5 (20) gave the highest and most uniform folic acid values. This method has been used to liberate folic acid from rat livers (25) and Luckey *et al.* (21) have also found that takadiastase digestion was satisfactory for the hydrolysis of muscle tissues for folic acid analysis. Wright *et al.* (22) reported that takadiastase treatment increased the amount of folic acid liberated from rat tissues as compared with the values obtained for untreated samples. In the present work a 1 g. sample of tissue was homogenized by means of a Potter-Elvehjem homogenizer or Waring blender prior to enzyme treatment. The folic acid content of muscle and organ tissues is expressed as the B<sub>12</sub> potency per 100 g. of undried meat (Table I).

The percentage retention of folic acid, calculated on the basis of the vitamin content of the entire cut of meat before and after cooking or curing, as described by Schweigert *et al.* (2, 4), is summarized in Table II. These figures were calculated on the basis of both the *S. faecalis* and *L. casei* determinations. The drippings from the cooking tests were not available for assay, consequently only the percentage retention in the meat was determined.

TABLE II

*Percentage Retention of Folic Acid After Cooking and Curing Meats*

Type of meat	Method of cooking	Percentage retention of folic acid	
		<i>S. faecalis</i>	<i>L. casei</i>
Veal leg	roasting	26	44
Veal shoulder	roasting	27	35
Lamb leg	roasting	9	22
Lamb leg	roasting	23	46
Veal sirloin chop	braising	10	31
Veal shoulder chop	braising	8	10
Veal flank	stewing	9	21
Pork ham	roasting	19	36
Pork ham	roasting	16	—
Pork ham	curing	67	65
Pork ham	curing	65	—

## DISCUSSION

As shown in Table I, the folic acid content of liver and kidney is considerably higher than that obtained for beef, pork, lamb or veal muscle. Veal muscle tissue and beef round were somewhat higher than the pork or lamb muscle tissues analyzed. The values obtained for veal muscle with *L. casei* were somewhat lower than those obtained

with *S. faecalis*. However, comparable values were obtained with both *S. faecalis* and *L. casei* for most of the other samples tested. Cheldelin and Williams (23) have reported somewhat higher folic acid values for a few muscle and organ tissues analyzed after a similar digestion procedure. Improvements in the basal media in the method of Teply and Elvehjem (19) that were used in the present work and the use of vitamin B<sub>6</sub> rather than solubilized liver as a standard may account for the differences noted.

The percentage retentions observed after cooking and curing meats are of the same magnitude as those reported by Cheldelin *et al.* (24). These workers reported from 5 to 40% retention of folic acid after frying and steaming pork loin or ham and veal chops. A 12% retention was observed after roasting mutton shoulder. In the present work from 8 to 27% of the folic acid was retained after roasting, braising and stewing procedures as measured by *S. faecalis* and from 10 to 46% as measured by *L. casei*. The retention was somewhat higher in most cases when measured by the latter organism. A higher retention of folic acid was observed after curing pork hams than after cooking. Although the drippings from the cooking tests were not available for assay, it is doubtful if an appreciable quantity of folic acid was present, since this vitamin is very labile.

The retention of the B vitamins after cooking and processing meats varies markedly. In previous work and in the present report it has been shown that choline, nicotinic acid and riboflavin are stable to cooking procedures and thiamine, biotin, vitamin B<sub>6</sub> and folic acid are more easily destroyed.

### SUMMARY

1. The folic acid content of muscle and organ tissues has been determined by the use of both *S. faecalis* and *L. casei*.
2. Liver and kidney were found to be the richest sources of folic acid. Veal muscle tissue was somewhat higher than pork or lamb.
3. From 8 to 46% of the folic acid was retained in the meat after cooking and 65% was retained after curing pork hams.

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# Changes in Polyene Synthesis Induced by Mutation in a Red Yeast (*Rhodotorula rubra*)

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## INTRODUCTION

The present report describes experiments on the pigmentation of a microorganism which were undertaken to gain, if possible, some insight into the mechanism of the biosynthesis of polyenes. For this purpose an intensely pigmented yeast, *Rhodotorula* (*Torula*) *rubra*, has been used.

Previous studies on the formation of polyenes in higher plants have been mainly limited to investigations on the influence of nutritional or other environmental conditions. Light increases the amount of carotenoids in etiolated seedlings (20) but is not, in general, essential to the formation of carotenoids, *e.g.*, in carrots. Absence of oxygen or temperatures above 30°C. hinder the color development in the ripening tomato (22, 4, 21). Production of polyenes is genetically controlled in the tomato. Fruits carrying a certain dominant gene produce lycopene (and minor red pigments) as well as some yellow carotenoids. In contrast, fruits homozygous for the recessive gene build up only the yellow pigments (13).

Only scattered observations on the factors influencing polyene formation in microorganisms are available. According to Fromageot and Tehang (7) the pigmentation of *Rhodotorula Sanniei* remains qualitatively the same within the temperature limits of 14°C. and 28°C. Lederer (12) found that light is needed for the full development of the *Rhodotorula* (*Torula*) *rubra* pigments. The carotenoids of purple bacteria have most recently been investigated by van Niel and Smith (15), Karrer and Solmssen (11), Polgár, van Niel and Zechmeister (17), and other authors (*cf.* 18). This field has been critically reviewed by van Niel (16).

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\* Contribution No. 1036.

It is well known that many biochemical reactions are controlled by specific genes. In *Neurospora*, Beadle and Tatum (2) and Beadle (1) have established the genetic control of the biosynthesis of numerous compounds. Mutation of an individual gene leads to alterations in the ability of the organism to carry out a specific chemical process.

The artificial production of mutants has been accomplished by a number of different methods, for example, by ultraviolet irradiation (5; cf. 3). By the use of this technique we have obtained seven mutant strains of *Rhodotorula* varying in color from red through brownish-yellow to white. The composition of the polyene formed by the original strain as compared with those of the mutant strains was then studied by chromatographic analysis.

### THEORETICAL

The composition of the *Rhodotorula rubra* pigments depends on the particular strain and on cultural conditions and, for this reason, earlier investigators have obtained somewhat varying data. All agree, however, on the presence of two components, viz., the intensely red torulene,  $C_{42}H_{58}O_2$ , and the orange  $\beta$ -carotene,  $C_{40}H_{56}$ , both isolated from the organism in pure form by Lederer (12). This investigator further detected a labile polyene hydrocarbon pigment and a carotenoid of acid character. His observations were, on the whole, confirmed by Fink and Zenger (6) who were, however, unable to obtain the acid pigment in all cases. An important study of a variety of *Rhodotorula Sanniei* was reported by Fromageot and Tchang (7). In chromatographic experiments these authors resolved the pigment into one acid and seven neutral components, three of the latter occurring only in traces. The four major hydrocarbon-carotenoids were identified as lycopene,  $\beta$ -carotene,  $\gamma$ -carotene and torulene, of which the two latter were obtained in crystalline form. The acid pigment is probably identical with that found by Lederer (12) in *Rhodotorula rubra*. According to Fromageot and Tchang, cultures grown with glycerol as a carbon source produce more intense pigmentation than cultures grown with glucose (in the presence of vitamin B<sub>1</sub>). Recently, Karrer and Rutschmann (10) have carried out large scale experiments with *Rhodotorula*. They were successful in isolating the acid pigment in crystalline form, and named it "torularhodin" (probably,  $C_{37}H_{48}O_2$ ). It constituted the main pigment in their material as it was in that of Fromageot and Tchang (7).

In our cultures no carotenoids with acid character occurred in any marked quantity. The extracted *Rhodotorula* pigment was resolved on the Tswett column essentially into four fractions, viz., torulene (about 76% of the total carotenoids);  $\beta$ -carotene,  $C_{40}H_{56}$  (11%);  $\gamma$ -carotene,  $C_{40}H_{56}$  (9%); and an unidentified carotenoid, "pigment A" (4%). When partitioned between 90% or 95% methanol and petroleum ether, all these pigments show epiphasic behavior. In each of our colored

mutant strains, the same four principal pigments were found as mentioned above for the original culture. In only one mutant (II) did an additional pigment ("B") occur in moderate quantities (11%). This pigment may possibly be present also in traces in other mutants.

The results of the quantitative chromatographic resolution of the respective pigment mixtures and the corresponding spectral data are summarized in Tables I-II.

TABLE I  
*Approximate Quantities of Individual Polyenes Contained in  
Rhodotorula Rubra Mutants*  
(The figures give mg. of polyene/100 g. of dehydrated yeast)<sup>1</sup>

Mutant	Macroscopic color of the culture	Torulene	Pigment "A"	$\gamma$ -Carotene	Pigment "B"	$\beta$ -Carotene	Phytofluene	Sum of polyenes
Original	red	5.2	0.40	0.74	—	0.76	0.63	7.73
Original	red	5.2	0.16	0.55	—	0.71	0.75	7.37
VII	red	6.4	0.44	2.1	—	1.3	1.1	11.34
IV	red	6.6	0.56	3.1	—	2.4	1.2	13.86
VI	red	6.0	0.51	2.2	—	2.0	1.3	12.01
II	brownish-orange	0.30	0.88	2.9	0.60	0.95	0.75	6.38
I	pale orange	0.16	0.22	0.80	—	0.64	1.0	2.87
III	colorless	0.02	—	—	—	—	—	0.02
V	colorless	—	—	—	—	—	—	0

<sup>1</sup> The pigments are listed in the order of decreasing adsorption affinity. Pigments "A" and "B" were evaluated as " $\alpha$ -carotene". The quantity of torulene was estimated on the basis of an extinction curve given by Lederer (12). The value employed for torulene in hexane at 484 m $\mu$ . is,  $E_{1\text{cm.}}^{\text{mol.}} = 16.0 \times 10^4$ .

Table I shows that, in those mutants which are visibly much less colored than the original, this is due primarily to restricted torulene synthesis. For example, the brownish-orange mutant II contained roughly as much total pigment as the original strain but produced only  $\frac{1}{17}$  as much torulene; the absolute quantities of all the other pigments were increased in this case.

Table II includes some data concerning phytofluene. This colorless polyene-hydrocarbon with isoprenic structure (perhaps  $C_{40}H_{64}$ ) is characterized by a typical spectral curve and, in ultraviolet light, by



TABLE II

*Spectral Characteristics of the Polyenes Observed in Rhodotorula*

(The figures in parentheses are those observed on addition of iodine. In the spectrophotometer readings, italicized wave lengths designate the maxima)

Pigment	Visually observed maxima		Maxima and minima observed in the Beckman spectrophotometer
	in CS <sub>2</sub> (m $\mu$ )	in petroleum ether (m $\mu$ )	in petroleum ether (m $\mu$ )
Torulene	564, 522, 488	520, 486, 454.5 (517.5, 484, 452)	519, 505, <i>486</i> , 466, <i>460</i>
$\gamma$ -Carotene	532.5, 495, 460	495, 461.5, 433.5 (492, 458.5)	<i>493</i> , 479, <i>462</i> , 444, <i>437</i>
$\beta$ -Carotene	519, 484, 452	485, 453, (483, 450)	<i>480</i> , 469, <i>453</i>
Pigment "A"	503, 470, 440	469.5, 441.5 (468.5, 439)	<i>470</i> , 457, <i>440</i> , 425, <i>415</i>
Pigment "B"	455, 424	425.5	<i>424</i> , 411, <i>399</i> , 386, <i>377</i>
Phytofluene	—	—	<i>367</i> , 358, <i>348</i> , 338, <i>332</i>

an intense greenish-grey fluorescence of its solutions or adsorbates (23). Phytofluene which appears to be involved in carotenoid biosynthesis (24) is common in higher plants where it was found particularly in carotenoid-rich but chlorophyll-free tissues. The samples of baker's yeast investigated did not contain the compound. The present experiments show that considerable quantities of phytofluene occur in those *Rhodotorula* cultures which produce substantial amounts of pigment. Thus, as is shown in Fig. 1, the presence of chromophores of very varying length can be demonstrated in the red yeast.

In such mutants as those (III, V) in which the pigment synthesis is completely (or practically) blocked, no phytofluene was found. However, the biosynthesis of carotenoids was accompanied by the formation of phytofluene (mutants I, II, IV, VI, VII). The pale-orange mutant I contained less than  $\frac{1}{3}$  as much total pigment as the original strain but contained noticeably more phytofluene. These data may be interpreted

on the assumption that phytofluene is an intermediate in pigment biosynthesis, and that it may accumulate to a limited extent when the process leading to its conversion to pigment is partly blocked. Final evidence as to the participation of phytofluene in pigment synthesis is not given by the present experiments.

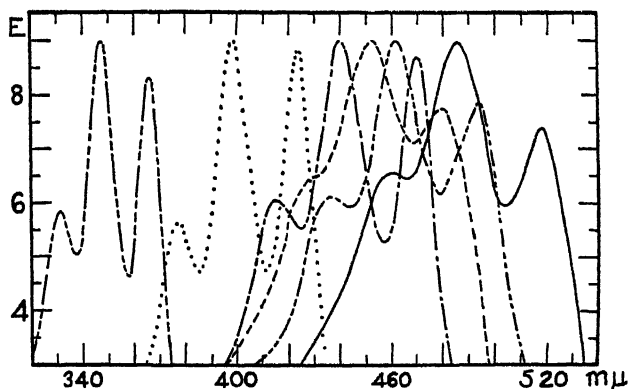


FIG. 1

Extinction Curves (in Petroleum Ether) of the Chromophores  
Biosynthesized by *Rhodotorula rubra* and Some Mutants

—, Torulene; ·····,  $\gamma$ -Carotene; — — —,  $\beta$ -Carotene; — · — ·, Pigment A;  
· · · · ·, Pigment B; and — · — · — ·, Phytofluene

Our mutant strains fall then into three categories. (a) In the strains IV, VI and VII, the total polyene accumulation is greater than in the original; whereas the torulene content is increased only 15–25%, the carotenes increased by 2–4 times. (b) In strains I and II the amounts of the yellow carotenoids are normal or above normal but the red pigment content is greatly diminished. (c) Finally, in III and V not more than negligible traces of any pigment are formed.

In the colorless mutants it would appear that a basic general process leading to pigment formation (possibly the production or conversion of an unknown precursor) has been interfered with in an early stage. On the other hand, in mutants I and II the production of a specific pigment is checked while that of the others remains unimpaired. These features may be tentatively summarized as follows:

<sup>1</sup> This solution contained per 100 ml.:  $\text{MnSO}_4$ , 2.0 g.;  $\text{ZnSO}_4$ , 0.22 g.;  $\text{CuSO}_4$ , 0.08 g.;  $\text{MoO}_3$ , 0.02 g.;  $\text{H}_2\text{BO}_3$ , 2.9 g.

in light are intensely red (12). All cultures were allowed to develop in diffuse daylight, although the above observation of Lederer was not in general confirmed with our strains.

*Induction of Mutations.* Mutations were induced by irradiation of cell suspensions with ultraviolet light. For this purpose a small quantity of yeast was removed from a stock culture and suspended in sterile water of a volume sufficient to yield a concentration of ca. one thousand cells per ml. The suspension was then transferred to syracuse dishes, 1 ml. per dish. These dishes were exposed to the radiation of a 20 inch quartz mercury vapor lamp (Westinghouse "sterilamp") at a distance of approximately 5 cm. During the exposure time of 4 minutes the thin layer of cell suspension in each dish was agitated with a sterile glass rod to increase the uniformity of exposure of individual cells. After this exposure, the 1 ml. of suspension in each dish was plated out in the synthetic medium. The conditions used resulted in the growth of 10-20 colonies per plate, corresponding to a survival of only 1-2% of the original cells. When the colonies had attained a diameter of 1-2 mm., the plates were inspected for the appearance of colonies having colors different from the parent strain. Of the aberrant colonies only those which appeared indistinguishable from the parent in microscopic morphology and in colony form and growth characteristics were taken for further work. From 600 exposed plates, or roughly 9000 individual irradiated cells, seven new strains differing in color from the original were obtained which varied from red through yellow to completely colorless. Each strain as it was found was subjected to two consecutive plating out procedures.

No genetic test to establish the mutant nature of these strains unqualifiedly can be made in *Rhodotorula*, and the suspicion that the aberrant strains might represent chance contaminants unrelated to the original strain could be entertained. The close resemblance of each aberrant strain to the original in morphological and cultural details as well as the conditions under which the aberrant strains were obtained lead us, however, to refer to these seven strains as mutants.

*Mass Cultures.* Various methods for the production of large quantities of cells were investigated. Variable growth, with generally poor pigment formation, occurred in aerated liquid cultures whereas uniformly well-pigmented cultures were obtained on agar medium. As culture vessels pint wine flasks of the flat whiskey type were used. Each flask, containing 70 ml. of medium, was placed flat so as to provide the maximum culture surface. The flasks were inoculated with a heavy suspension of the desired strain and incubated at 23-24°C. under sterile conditions. The original and the seven mutant strains were placed in randomized blocks and the flasks were shifted in position at weekly intervals to minimize possible inequalities in illumination. Two main experiments were carried out with 20 and 40 flasks for each strain, respectively. The cultures were grown for 20 to 40 days before harvest.

*Extraction of the Polyenes.* The yeast was scraped from the agar by means of a glass spatula. The agar should not be scratched and, if it is soft, ice cooling is recommended. The material (about 100 g.), obtained from 40 bottles, was mixed and its dry weight determined in an aliquot sample (Abderhalden apparatus, boiling acetone, 0.1 mm. pressure). The main bulk of the wet yeast was ready for the extraction. It is, however, difficult to achieve complete removal of the polyenes by current methods. For example, when the culture is rubbed with sea sand in a mortar, in the presence of methanol and petroleum ether, only a fraction of the pigment passes into the solvents. Satisfactory extraction can be obtained after a thorough destruction of the cell walls with strong alkali as follows.

The material was treated in a 500 ml. glass-stoppered Erlenmeyer flask with 200 ml. of 20% methanolic KOH and 100 ml. of benzene and was shaken mechanically for 1-1.5 hours, whereby a thick suspension of yeast material appeared. As the material stood for 15-20 hours, this emulsion separated but reformed on brief shaking. The emulsified liquid was then centrifuged in two 250 ml. bottles for a few minutes (International Centrifuge, Size I, Type SB; 2000 r.p.m.). Three layers appeared: the colored benzene solution at the top, followed by methanolic alkali while the yeast residue remained at the bottom (in some cases the yeast floated in the middle). The benzene solution was pipetted out and the remainder re-extracted twice with fresh benzene (20 min. of mechanical shaking, then centrifuging). After this treatment the *Rhodotorula* residue appeared colorless.

The pigment solution (300-400 ml.) was divided into four parts using 250 ml. centrifuge bottles. On addition of 1 vol. of water a thick emulsion appeared from which the benzene layer could be separated in the centrifuge. This emulsion was extracted with fresh benzene. The combined pigment solution no longer showed a tendency to form emulsions in the presence of water. It was washed alkali-free in a continuous apparatus (13), dried with sodium sulfate and evaporated to dryness *in vacuo* in the presence of a slow stream of nitrogen. To free the residue entirely from benzene, the evaporation was repeated after addition of petroleum ether (b.p. 60-70°). The residue was oily.<sup>1</sup>

*Separation of the Pigments and of Phytofluene.* The above-mentioned oil was dissolved in 20 ml. of petroleum ether and adsorbed on a 17 × 1.9 cm. column, composed of 4 parts of calcium hydroxide (Shell

<sup>1</sup> It is not advisable to repeat the saponification at this stage. While such an operation would remove most of the oil, the losses caused by emulsions (which prevent extraction of the pigment) would be heavy.

Brand lime, chemical hydrate, 98% through 325 mesh) and 1 part of alumina (Alorco, Grade F, minus 80 mesh). When such a column is developed with petroleum ether containing 7.5% acetone,  $\beta$ -carotene and phytofluene, mixed with large amounts of oily impurities, are washed down into the filtrate while the other pigments form a well defined chromatogram (Table IV). Each zone was eluted with alcohol. After a transfer with water into petroleum ether it was ready for examination in the Zeiss grating spectroscope (visual) and the Beck-

TABLE IV  
*Characteristic Chromatograms of Resolved Pigment Mixtures  
Obtained from Some Rhodotorula Mutants*

( $\beta$ -Carotene and phytofluene were contained in the chromatographic filtrates, except Mutant III. The figures denominate the width of the zones, in millimeters, on a calcium hydroxide-alumina column,  $17 \times 1.9$  cm.)

<i>Original (red)</i>	<i>Mutant VII (red)</i>
30 pale pink (traces)	30 pale pink (traces)
15 dark red (torulene)	10 dark red (torulene)
6 red (neotorulene)	10 red (neotorulene)
8 pale yellow (pigment A)	10 yellow (pigment A)
30 colorless	22 orange ( $\gamma$ -carotene)
30 orange ( $\gamma$ -carotene)	
<i>Mutant IV (red)</i>	<i>Mutant VI (red)</i>
20 pale pink (traces)	30 pale pink (traces)
12 dark red (torulene)	10 dark red (torulene)
5 red (neotorulene)	4 red (neotorulene)
8 yellow (pigment A)	6 yellow (pigment A)
30 colorless	20 colorless
35 orange ( $\gamma$ -carotene)	20 orange ( $\gamma$ -carotene)
<i>Mutant II (brownish-orange)</i>	<i>Mutant I (orange)</i>
10 almost colorless (traces)	24 almost colorless (traces)
5 dark red (torulene)	10 dark red (torulene)
5 colorless	5 pink (neotorulene)
4 red (neotorulene)	3 colorless
1 colorless	15 yellow (pigment A)
12 yellow (pigment A)	10 colorless
25 orange ( $\gamma$ -carotene)	20 orange ( $\gamma$ -carotene)
50 colorless	
15 pale yellow (pigment B)	
<i>Mutant III (colorless)</i>	
20 colorless	
3 light pink (torulene)	

man photoelectric spectrophotometer. Such an examination was repeated after the corresponding pigments, as obtained from different mutants, were combined and rechromatographed to eliminate minor contaminants.

The chromatographic filtrate containing  $\beta$ -carotene and phytofluene was evaporated and the residue submitted to the same alkaline treatment as described above. This resaponification process was so effective that a sharp chromatogram could be obtained under the conditions described but using petroleum ether with only 2% acetone as a developer. Below minor top zones a broad  $\beta$ -carotene zone appeared followed by phytofluene, the adsorbate of which fluoresced strongly in ultraviolet light. These fractions were eluted and submitted to spectroscopic study.

*Identification of the Polyenes.* The pigments studied were characterized by their spectra (Table II). In the case of phytofluene the adsorption behavior and the very typical extinction curve were conclusive (maxima at 367, 348, 322  $m\mu$  in hexane). Torulene showed in benzene the visually observed maxima: 537, 499.5, 466.5  $m\mu$ .

Spectroscopically and chromatographically corresponding pigments obtained from different mutants were shown to be identical by mixed chromatogram tests. Furthermore,  $\gamma$ - and  $\beta$ -carotene were mixed-chromatographed using authentic samples which originated from other sources. All polyenes occurring in the *Rhodotorula* showed an entirely epiphasic behavior.

"Pigment A" possesses maxima at shorter wave lengths than  $\alpha$ -carotene, but its adsorption affinity surpasses even that of  $\gamma$ -carotene. Its maxima in  $CS_2$  would correspond to those of  $\beta$ -carotene-diepoide as described by Karrer and Jucker (9) but we were unable to reproduce with our material the wave length shift of the maxima upon a treatment with HCl-containing chloroform. With regard to "Pigment B" it should be noted that the exceptionally short wave lengths of its maxima in  $CS_2$  (Table II) lie near those reported by Karrer (8) for auroxanthin. Nevertheless, no identification can as yet be claimed in this case either.

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## SUMMARY

A red yeast, *Rhodotorula rubra*, was induced by ultraviolet illumination to mutate and the polyene mixture extracted from each of seven mutants was submitted to chromatographic analysis. The colored mutants contained torulene,  $\gamma$ -carotene,  $\beta$ -carotene and an unidentified pigment in different proportions, while the macroscopic color of the cultures varied from intense red to colorless. All pigmented mutants also contained phytofluene, a colorless strongly-fluorescing polyene, possibly a precursor of carotenoids. The biogenesis of the red yeast polyenes is discussed.

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# The Content and Biological Availability of Carotene in Dehydrated Carrots Stored at High Temperatures

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## INTRODUCTION

The method of dehydration and manner of packaging and storage may greatly modify the desirability of a dehydrated food. The dehydration process itself should yield a product which, when reconstituted with water, is as close as possible to the fresh product in appearance, taste, and nutritive value. Proper packaging and storage of the product should minimize the loss of these qualities. One approach to the storage problem is to find out what happens when a dehydrated food is kept under conditions which accelerate the deteriorative changes. Accordingly, a study was made of samples of dehydrated carrots stored under adverse conditions. The experimental details to be given are those of assay number 8 reported briefly in another paper (1).

## EXPERIMENTAL

Commercial dehydrated carrots were stored under a variety of conditions as part of a comprehensive experiment. The samples considered here contained 5% moisture and were packed in No. 2½ friction-sealed tin cans. Cans were removed periodically for sampling and analysis. Two series of samples were selected for further study. One of these, stored at 98°F., showed the usual rate of carotene destruction

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as judged by chemical analysis, while the other, stored at 120°F., revealed less destruction of carotene.

The carotene content of the samples examined was determined by chromatographic analysis of the pigments and subsequent spectrophotometric determination as previously described (2).

It was previously reported by Beardsley, Prindle and Stevens (3) that storage at 98°F. and 70°F. showed no consistent effect of temperature on stability of carotene, and Heberlein and Clifcorn (4) found that storage temperatures of 98°F. and 130°F. were detrimental to carotene. However, for the particular samples discussed herein, storage above 98°F. (120°), instead of hastening the destruction of carotene, appeared to retard or stop it, as shown in Table I. Other characteristics

TABLE I  
*Change in Carotene Content of Dehydrated Carrots During Storage*

Storage Time Days	Carotene content with storage at	
	98°F. (sample No. 18) mg./100 g.	120°F. (sample No. 12) mg./100 g.
0	111	111
125	100	105
254	84.5	107
425	69	94

of the dehydrated carrots had changed and the degree of change was greater at the higher temperature. The carrots had darkened greatly, they had a molasses-like odor and the taste bore no resemblance to that of carrots. Although the chromatographic adsorption-spectrophotometric method of analysis was considered to be specific for carotene, it was deemed advisable to test this anomalous preservation of carotene at elevated storage temperatures by bioassay. This was done on two samples stored for 425 days at 98°F. and 120°F.

The biological availability of the carotene was estimated by the growth response of vitamin A-depleted rats as described previously (1). The positive control rats for the bioassay received a standard supplement consisting of S.M.A. crystallized  $\beta$ -carotene dissolved in cottonseed oil. This standard was fed at three levels. All other supplements were fed at four levels. Two solutions of the standard were used: one of low concentration and the other containing five times as much carotene. In this manner the volume of oil was not excessive for

those animals receiving the larger amounts of supplement. This precaution was deemed advisable to avoid the possibility of unduly favoring the absorption of carotene. The carotene for the standard was weighed and dissolved in petroleum ether; cottonseed oil was added, the petroleum ether was removed by vacuum and the solution was made up to volume with oil. The concentration of carotene in the final solution checked spectrophotometrically ( $\alpha$  436<sub>mμ</sub> = 199) (5) with the value obtained by weight.

Each sample of dehydrated carrots was ground and divided into two parts. One part was subdivided into a number of vials and stored at -30°F. A fresh vial was used each week for the supplementary feedings in the bioassay, the vial being kept in the freezing compartment of a kitchen-type electric refrigerator for the three days between supplements. The other portion of each sample was extracted with acetone. This extract was purified by chromatographic adsorption on dicalcium phosphate and analyzed spectrophotometrically. The extract was added to cottonseed oil in the manner previously described for the standard so as to give known concentrations as determined by spectrophotometric analysis. Two concentrations of each extract in cottonseed oil were used as described for the standard. The five supplements fed to the rats were, then, the standard  $\beta$ -carotene in oil, solid samples No. 12 and No. 18, and oil solutions of the extracts of these samples.

The results of the assay with standard errors of the means are shown in Table II. The chemical determination showed a much greater re-

TABLE II  
*Carotene in Stored Dehydrated Carrots*

Product	Storage temp. °F.	Dried Product as received	Extract of Product	
		Bioassay mg./100 g.	Chemical mg./100 g.	Bioassay mg./100 g.
Sample No. 12	120	27±3.8	94	81±9.5
Sample No. 18	98	23±3.5	69	60±7.1

tention of carotene at 120°F. than at 98°F. The bioassay of the extracts gave values which, while slightly lower, were well within the experimental limits of the assay. That is, the bioassay agreed with the chemical assay when extracts of the samples were used. However, with the dehydrated material, growth of the animals indicated that only a

third, or less, of this carotene was being utilized. The differences between the results on the dehydrated products and those on the extracts are highly significant as shown by the standard errors of the means.

During the course of the assay it was noted that the animals on the lower dosages of dry supplement were responding more poorly than would be expected as judged by the rats receiving greater amounts of the same materials. In the calculations this difference became even more noticeable. Table III presents the slopes of the log dose-response

TABLE III  
*Slopes of Log Dose-Response Curves*

	Solid Supplement		Oil Supplement	
	<i>Male</i>	<i>Female</i>	<i>Male</i>	<i>Female</i>
Standard	—	—	41.2	18.9
No. 12	70.7	41.9	42.4	15.5
No. 18	86.4	32.9	45.6	23.1
Mean	78.5	37.4	43.1	19.2

curves. The greater slopes found for the animals receiving the solid supplements indicate poorer utilization, especially at the lower dosage levels. If the data from rats on the lowest dosage levels of solid supplement were eliminated from the calculations, the slopes of the curves would more nearly approach those for the oil supplements. Despite the anomalous behavior of these rats, their inclusion in the calculations did not affect the carotene values substantially and therefore the calculations were based on data from all of the animals.

## DISCUSSION

It might have been anticipated that the rate of carotene destruction would have been accelerated at elevated temperature. That the opposite was true in these particular instances was a most unexpected, and perhaps exceptional, finding. It is possible that the protection of the carotene was indirect. The high temperatures caused discoloration (caramelization or aldehyde-amine reaction) of the dehydrated carrots. Any reducing substances formed (*e.g.*, reductones) may have acted as antioxidants, protecting the carotene from destruction.

A comparison of the chemical method of estimation of carotene

with the bioassay procedure indicated that: (a) the extracts contained carotene in the amounts indicated by the chemical method—that is, the bioassay and the chemical methods agreed as well as could be expected; and (b) the solid material definitely did not give rats the protection that might have been expected on the basis of carotene content. Although the carotene of freshly dehydrated carrots may not always have the high biological availability of the carotene in raw carrots, we have never found the availability to be as low as it was for the high-temperature-storage products. The high temperature has modified the physical characteristics of the carrots so as to prevent complete absorption of the pro-vitamin, or else the carotene has been converted to one or more isomeric forms having lower vitamin A potency. If the latter interpretation is correct the solvent extraction procedure must permit reconversion of the carotene to the isomer of greater biological availability.

From a nutritional point of view the most significant conclusion to be drawn from the results reported in this and the preceding paper (1) is that the biological availability of the carotene as measured by bioassay is more important than the carotene content of a product as revealed by a chemical analysis. This conclusion is in harmony with the point of view expressed in a recent editorial (6) summarizing studies on the availability of iron by Sherman, Elvehjem and Hart (7) and Nakamura and Mitchell (8), of calcium by McCance and Widdowson (9), and of thiamine by Parsons, Williamson and Johnson (10).

### SUMMARY

Dehydrated carrots stored at 98°F. and 120°F. in an atmosphere of CO<sub>2</sub> were changed so as to be unacceptable as a food from an aesthetic and organoleptic point of view, but the carotene content was not lowered at 120°F. as much as it was during storage at 98°F. or lower.

It is suggested that the partial destruction of the carrots at the elevated temperature created conditions unfavorable to the oxidation of carotene.

The spectrophotometric analysis and the bioassay of the extracts of these products agreed quite well. However, when the solid material was used for bioassay, less than one-third of the contained carotene was available to the rat.

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# The Content and Biological Availability of Carotene in Raw and Dehydrated Carrots and Other Vegetables

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## INTRODUCTION

During the last few years considerable attention has been given to the chemical determination of carotene in fresh and dehydrated vegetables, especially carrots, and to the bioassay method for evaluating the vitamin A potency of these materials. Much painstaking effort has been expended in trying to discover the cause or causes of the apparent discrepancy found at times between the chemical and the bioassay methods. The data presented in this paper indicate that each method is fundamentally sound. When properly applied, the chemical method measures the amount of carotene present. And also when properly applied, the bioassay method measures the biological availability of that carotene as a source of vitamin A. We should not expect the two methods to agree where conditions of processing or storage may decrease the availability of the carotene present. This paper presents evidence suggesting this view.

The use of crystalline  $\beta$ -carotene as the international standard for the biological assay of vitamin A activity is based on the fact that certain carotenoids, especially  $\alpha$ - and  $\beta$ -carotene, can be converted to vitamin A by the animal body. However, it does not follow that ingested carotene is always and quantitatively changed into vitamin A. There are many opinions as to how successful this conversion may be, and there exist numerous ways of modifying the form of the supplement

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or type of diet to change the availability of the provitamin. In general it would seem that the majority opinion is as follows: Absorption of carotene is more nearly quantitative with low than with high intakes. Carotene dissolved in vegetable oil is more readily available than it is when in its natural dispersion in solid foods. A low-fat diet decreases the absorption of carotene. The widely divergent values reported in the literature for availability of carotene are probably due in part to differences in: (a) species (rat, chicken or man), (b) amount of supplement, which has varied from minute amounts to "lavish" intakes, (c) type of diet, the diet frequently not being specified, or else described as "adequate," (d) form of supplement, such as carotene in oil, or in water suspension, or in any one of a variety of natural products containing more or less pro- or anti-oxidants (*e.g.*, vitamin E), and (e) criteria on which judgment is based.

Illustrative of the effect criteria of judgment may have upon the conclusion reached is the concept that absorption is the difference between intake and output of carotene. This assumes that there is no destruction of carotene in the alimentary tract, and is supported by evidence that carotene mixed with feces and incubated can be recovered completely (1). Such data in turn assume that the methods for the estimation of the carotene are adequate, and this is probably not always the case. This was shown by Kemmerer and Fraps (2), who found yellow non-carotene pigments in feces. These workers showed later (3) that the pigment was not adsorbed by magnesium hydroxide under conditions which did cause adsorption of carotene and they suggested that this might be an aid in the estimation of carotene in feces.

During the course of the work reported in the present paper, feces were collected daily for a week from three groups of rats: negative controls receiving no carotene supplement, positive controls given four international units of carotene per rat daily, and assay animals receiving a daily supplement of six mg. of dehydrated carrot per rat. The feces were stored in nitrogen at about 0°C. and analyzed as soon as possible after the period of collection was completed. The pigment was extracted from the feces and subsequently adsorbed on dicalcium phosphate as described in the analytical method for the determination of carotene in vegetables (4). The absorption spectra of "carotene" solutions thus isolated from the rat feces were characterized by a broad continuous band which decreased in density with increasing wave length. Superimposed upon this broad band were slight absorption maxima characteristic of carotenoids. Therefore, if carotene in feces is estimated by yellow color alone, the reported excretion will be higher than the true values and carotene absorption will be judged to be poorer than is actually the case. The amount present was too small to permit quantitative estimation.

Absorbed carotene will cause a measurable response in an animal depleted of vitamin A. This fact is the basis of the bioassay method for the evaluation of the vitamin A activity of the carotenoids. The

absorbability of carotene in raw and dehydrated carrots, for example, can then be measured by the bioassay method, provided the amount of carotene ingested is known and the amounts fed are kept at the low level required for assay of vitamin A potency. The use of the bioassay method for studying carotene absorption has the advantage that absorption is measured over a period of weeks on a large number of animals, and that conditions can be kept fairly constant throughout the assay and between different assays. The method has the disadvantage of being expensive, time-consuming and of being limited to sub-optimal intakes of the provitamin.

During the last several years the Western Regional Research Laboratory has been developing effective methods of dehydrating vegetables and of packaging and storing the dehydrated materials so as to give satisfactory products. Some of these products have been given to the Pharmacology Laboratory of the Bureau for bioassay as a check on the chemical analysis, or at least as a measure of the biological availability of the carotene. The present report deals with the results obtained on fresh and dehydrated carrots, and on certain other dehydrated vegetables.

### METHODS

The biological availability of the carotene was estimated by the growth response of vitamin A-depleted rats. The method was essentially that described by Coward (5). The vitamin A-free diet previously described (6) was modified to include yeast (eliminating the daily administration of a yeast supplement), and to exclude agar, giving the following composition: vitamin A-free casein, 20.8; cornstarch, 64.6; salt mixture (U.S.P. No. 2), 2.4; dried brewers' yeast (Anheuser-Busch strain (1), 7.5; hydrogenated vegetable oil (Crisco), 4.7; Viosterol, 0.024. The supplement for the positive controls was crystalline carotene dissolved in cottonseed oil. In the first 7 assays, the carotene<sup>4</sup> was a mixture of 80-85%  $\beta$ -carotene and the rest  $\alpha$ -carotene, in such concentration as to give 1 I.U. in each drop of solution, assuming that each molecule of  $\alpha$ -carotene equaled one molecule of vitamin A, and that a molecule of  $\beta$ -carotene became two molecules of the vitamin. For assay 8, a solution of pure  $\beta$ -carotene was used.<sup>5</sup> Since the control and experimental rats received the same basic diet containing adequate

<sup>4</sup> Obtained from National Research Associates, Inc., South Whitley, Indiana.

<sup>5</sup> S. M. A. Corporation pure  $\beta$ -carotene.

amounts of tocopherol, the effect of this vitamin on carotene absorption as discussed by Hickman (7) is not a source of error. All carrot supplements were kept under nitrogen at 0°C., the dehydrated samples being ground to a powder and stored as above at the start of the assay period. The supplements, both positive control and unknown, were fed twice weekly for a 5-week period, at three or four levels of intake to groups of rats containing 8 or 9 animals each (11 in Assay 8). The animals were divided as to sex, litter and size at the start of the assay period, so as to make the groups as uniform as possible. Calculations of the dosage-response curves were made separately for males and females and the results weighted, according to the number of animals, to give the final values.

The standard error of the mean was always calculated for bioassay values. The variability of the chemical assay was not determined routinely, but was not more than 3% in the least-favorable materials tested. This percentage is considerably under the variation in bioassay, so that for all practical purposes the standard error of the mean for bioassay can be considered as the standard error of the difference between the chemical and the bioassay methods. If the difference amounted to at least 3 times the standard error, the difference was considered significant. This ratio of difference to standard error is given in the last column of Tables I and III.

## RESULTS

*Carrots.* The data on carrot products are presented in Table. I In all cases (except Assay 1) treatment of the carrot by blanching or dehydration appeared to increase the carotene content considerably over the value found for the raw carrot, as judged by chemical analysis. The cause of this apparent increase (amounting to 5-35%) during the process of dehydration has recently been ascribed to the loss of soluble solids in certain of the processing steps of washing and blanching (8). If any losses of carotene took place during blanching and dehydration they were obscured by the loss of soluble solids.

Assay 1 has been reported previously (6), although calculations were not on the basis of dry weight, as is the case in this paper. In Assay 2 the chemical and bioassay methods indicated an increased carotene content after dehydration, and a still higher value when blanching (*i.e.*, pre-cooking) preceded the dehydration.

TABLE I  
*Carotene Content of Carrot Products*

Carrot assay	Type of product	Carotene mg./100 g. dry wt.		Bioassay $\times 100$ Chemical	Variability of bioassay $\frac{100\sigma_x}{\bar{x}}$	Bioassay-chemical $\frac{\sigma_x}{\bar{x}}$
		Chemical	Bioassay			
1	Raw	82	100	122	14.7	1.5
	Blanched, dehydrated	77	75	97	15.6	0.2
2	Raw	130	86	66	16.0	2.1
	Raw, dehydrated	158	94	59	11.4	3.6
	Blanched, dehydrated	193	147	76	15.1	1.6
3	Raw	111	113	102	13.3	0.2
	1 min. blanch, dehydrated (Sample A)	149	84	56	11.3	3.9
	5 min. blanch, dehydrated (Sample B)	150	82	54	12.0	3.8
	10 min. blanch, dehydrated (Sample C)	163	122	75	12.0	2.1
4	2 months storage of Sample A	116	20	17	21.6	3.8
	Sample B	122	38	31	16.5	4.2
	Sample C	141	60	43	17.7	3.2
5	4 months storage of Sample A	91	18	20	23.7	3.4
	Sample B	101	61	60	20.4	2.0
	Sample C	114	62	55	19.5	2.3
6	8 months storage of Sample A	67	50	75	27.3	0.9
	Sample B	82	53	64	17.5	2.1
	Sample C	70	55	80	19.2	1.0
7	Raw	110	69	62	15.4	2.4
	Blanched	145	78	54	15.7	3.0
	Blanched, dehydrated	120	73	60	14.5	2.8
	Extract of raw	110	124	113	13.3	1.0
	Extract of blanched	145	193	133	15.1	2.2
	Extract of blanched, dehydrated	120	161	134	14.6	2.3
8*	Dehydrated, stored 14 months at 120°F. (No. 12)	94	27	29	13.9	5.1
	at 98°F. (No. 18)	69	23	33	15.3	4.4
	Extract of No. 12	94	80	85	11.7	1.3
	Extract of No. 18	69	62	90	11.8	0.9

\* Carotene values on samples No. 12 and No. 18 are reported on samples as received, because water content could not be determined readily.

Assays 3, 4, 5 and 6 were part of a large-scale experiment designed to cover several methods of preparation and the effects of storage on a single sample of carrots. Immediately after dehydration, chemical assay indicated an increased carotene content in the blanched dehydrated products, the values increasing with the duration of blanching. The carrots, after dehydration, were packed in air and stored at 90°F. Unfortunately, bioassays of the stored samples were not particularly satisfactory, since variations in response between animals in Assays 4, 5 and 6 were unusually large. However, the trend of the data for the bioassays indicated a much more rapid decrease in carotene content than did the chemical analysis; statistically significant differences between values by the chemical and the biological methods were found for all samples after 2 months' storage and for one sample after 4 months' storage. The data are shown more clearly in Fig. 1.

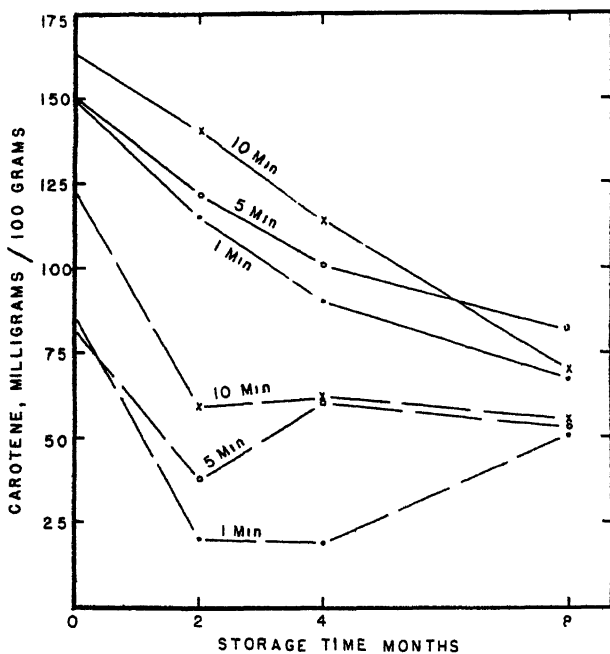


FIG. 1

The Effect of Storage on the Carotene Content of Dehydrated Carrots

Solid lines represent chemical analysis; broken lines are for bioassay. The three samples were blanched 1, 5 and 10 minutes, respectively, as indicated.

The steady decline in carotene content (chemical analysis) is apparent. Loss, as judged by bioassay, was very rapid during the first two months of storage, at the end of which time a low level was reached which remained more or less constant during the remainder of the 8-month storage period. In the bioassay curves, the apparent increases in activity for the product blanched for 5 minutes, between the second and fourth months, and for the 1-minute blanch between the fourth and eighth months, are of no statistical significance and presumably are due to the inherent variability of a bioassay technique.

In Assay 7, the products were fed to the rats in the form in which they were received, and also extracts prepared for chemical evaluation were dissolved in cottonseed oil and fed as supplements. The bioassay values of the extracts were about twice those obtained on the original products. The calculated carotene contents based on the bioassays were above those obtained by chemical analysis but the differences do not seem significant.

Assay 8 is reported in more details in another paper (9). The samples used in this assay were stored, dehydrated carrots which seemed by bioassay to contain very much less carotene than was indicated by chemical means. The conditions of storage were drastic and may be presumed to exaggerate loss in availability of carotene. Within the variability of the bioassay, the two methods agreed on the carotene contents of extracts of these products.

Since different batches of carrots are represented in Table I, and the blanching, dehydrating and storage methods were varied so as to find the most satisfactory procedures, the results must be discussed in a somewhat generalized manner. With these limitations in mind, it is possible to group the assays into six categories, as in Table II.

With raw carrot there was quite fair agreement between animal and chemical determinations in the 4 assays. In none of the assays was the difference between chemical and animal determinations statistically significant. A bioassay was made on only one sample of raw dehydrated carrots. The value obtained was 59% of the chemical value, and the difference appeared real. There also was but one sample of blanched carrots. Bioassay estimated the carotene content as 54% of the value obtained by chemical analysis, a significant difference. In other words, the carotene of these samples was not fully available to the rats.

TABLE II

*Comparison of Carotene Concentration in Carrots as Determined by Bioassay and Chemical Procedures*

Kind of carrot product	Number of assays	$\frac{\text{Bioassay} \times 100}{\text{Chemical}}$		Number of ratios significantly different from 100
		Mean value	Range	
Raw	4	88	62-122	0
Raw, dehydrated	1	59	—	1
Blanched	1	54	—	1
Blanched, dehydrated	6	70	54-97	2
Dehydrated, stored	11	46	17-80	6
Extracts	5	111	85-134	0

There were 6 samples of carrots which had been blanched and dehydrated. The bioassays averaged 70% of the chemical. Two of the six differences appeared real. When storage was added as another factor, the differences between the two methods became more marked. The bioassays on stored samples averaged 46% of the chemical analysis. Although there was considerable variation between animals in Assays 4, 5 and 6, it is still of interest that in 6 of the 11 cases the difference between the chemical and the bioassay methods appears statistically significant.

With the five extracts, there were two instances where the bioassay was slightly below the chemical and three where it was somewhat higher, but in no case was the difference significant.

*Other Vegetables.* In a study less intensive than that on carrot products, several other vegetable products were analyzed for carotene by both chemical and bioassay procedures. In only one instance (sweet potatoes) was the fresh material bioassayed. The results are included to show the order of agreement between the methods of assay and to indicate the amount of carotene which may be expected in various dehydrated vegetables. The data are presented in Table III.

Biological and chemical assays of dehydrated sweet potatoes, mustard greens, beets and green lima beans agreed with each other quite well. Dehydrated chard gave values by the two methods which were in fair agreement. The chard was stored in a closed metal container at room temperature. Six months later a chemical analysis showed that a considerable loss of carotene had occurred. Since this

loss was going on during the course of the assay, the agreement between the methods is probably better than the indicated 80%. Dehydrated beets showed no carotene by chemical analysis and could not be fed to rats in quantities large enough to protect them. The agreement for dehydrated corn was not good. If only the carotene content is considered, entirely neglecting the vitamin A value of cryptoxanthin, the biological value is still only about half of the chemical value. It may be that the rats are unable to absorb the carotenoids of the corn efficiently.

TABLE III  
*Carotene Content of Various Dehydrated Vegetables*

Type of Product	Carotene mg./100 g. dry wt.		Bioassay $\times 100$ Chemical	Variability of Bioassay $100\sigma_{\bar{x}}$ $\bar{x}$	Bioassay- Chemical $\sigma_{\bar{x}}$
	Chemical	Bioassay			
Sweet potatoes, fresh dehydrated	15.0	17.3	115	16.0	0.9
	>11.3	13.4	<119	17.3	<1.1
Mustard greens	53.2	50.2	94	20.3	0.3
Chard Repeat 6 mo. later	40.0	32.0	80	19.6	1.0
	28.8		111		0.6
Beets	0	0	—	—	—
Green lima beans	1.37	1.4	102	16.9	0.6
Corn Repeat 2 mo. later	0.5	0.26	<52	18.5	>2.9
	$\left\{ \begin{array}{l} \text{carotene} \\ +2.2 \\ \text{crypto-} \\ \text{xanthin} \end{array} \right\}$	0.27	<53	12.1	>3.1

### DISCUSSION AND CONCLUSIONS

The proper application of the chemical technique would seem to give a true picture of carotene content. It is also true that a careful bioassay will indicate the amount of utilizable carotene within the accuracy of the bioassay method. That the chemical and the bioassay methods do not always agree must be due to the inability of the test



animal to absorb all of the carotene in the supplement. When the carotene, which is poorly utilized in the dry product, is extracted and fed in cottonseed oil, the rat can use it. If knowledge of the carotene content of a sample is what is wanted, chemical assay would be the method of choice. If utilization of that carotene is the important question, chemical analysis may not give a true answer and the biological assay would be preferable.

On the basis of the above results, some generalizations appear warranted: The two methods of assay agree as well as can be expected when applied to raw carrots, although the results are somewhat inconclusive in two of the four experiments. The carotene of dehydrated carrots is not always fully available to the albino rat. Storage of dehydrated carrots leads to a gradual loss of carotene content as judged by both chemical analysis and biological assay.

Of the other dehydrated vegetables studied, there was substantial agreement between the two methods of assay for carotene, indicating that the rat can utilize this carotene. The one exception was corn, where utilization was poor.

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## Observations on the Inheritance of Latex Quality in *Cryptostegia*

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### INTRODUCTION

Rubber is generally found as an aqueous emulsion contained in specialized latex vessels of plants. Of the substances which accompany rubber in the coagulum of latex, many are also of the isoprene types such as gutta-percha, sterols, terpenes and other similar substances (1), all of which may be thought of as being constructed from a 2-methyl-2-butene or "isoprene type" polymer element. In spite of much work, little is known about the biological mechanism by which such compounds are synthesized.

A combined genetic and chemical approach affords an effective method for investigating some of the steps in the biosynthesis of related compounds. For example, workers at the John Innes Horticultural Institution (2) have found that gene action is evident through its control of the chemical configuration of flower pigments such as the position of oxidation, methylation and glucoside formation on an anthocyanin nucleus. Such data support the hypothesis of Horowitz (3) that "... each synthesis is controlled by a set of non-allelic genes, each gene governing a different step in the synthesis." Beadle and his associates (4) consider genes to control specific enzymatic reactions in *Neurospora*.

Latex systems have been observed in plants of many families, some of which are widely separated taxonomically. Latex vessels are considered to be living cells (1) and it is possible that much of their contents is synthesized within them. Latex vessels are very active in the production of the related hydrocarbons, gutta, rubber and triterpenes.

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Occurrence together of these substances in such specialized cells suggests that they are as related in formation as are flower colors.

Chemical analysis showed a great difference in the quality of latex obtained from young shoots of two species of *Cryptostegia*. *C. madagascariensis* latex contained a triterpene-ester as a principal constituent and practically no rubber (5). On the other hand, *C. grandiflora* latex contained much rubber and very little, if any, triterpene-ester. A study was made of the inheritance of latex quality in an  $F_2$  progeny derived from self-fertilization or sibbing of a natural hybrid, *C. grandiflora*.  $\times$  *C. madagascariensis*. Evidence is presented that latex quality is subject to relatively simple genetic control and, because of this finding, it is possible that both rubber and triterpene ester utilize a common precursor for their biosynthesis.

#### PLANT MATERIAL AND ANALYTICAL METHODS

*The Two Species of Cryptostegia.* All plant material used in this study was growing in the Plant Introduction Garden at Cocoanut Grove, Florida. *C. grandiflora* was grown from seeds collected in Florida, the western coast of Mexico and Arizona. Some of the plants were more than 19 years old while others represented new stock two and one-half years old. In spite of the difference in age and locality of origin, all the plants were very uniform in morphological and anatomical characteristics.

Twenty *C. madagascariensis* plants originated from 1924 seed collections made in Tanganyika Territory, Africa. A larger stock was derived from 1942 seeds of the African plants. Like *C. grandiflora*, all plants were remarkably uniform in plant characters. The material available for latex collections and cytological studies is considered fully representative of the two species of *Cryptostegia*.

*The  $F_1$  Hybrid.* The hybrid, *C. grandiflora*,  $\times$  *C. madagascariensis* was discovered in 1927. Quite by chance, a worker at the Plant Introduction Garden selected a follicle from a normal *C. grandiflora* plant which had been growing near a group of *C. madagascariensis* plants. After germination, it was soon apparent that the seedlings were different from either *C. grandiflora* or *C. madagascariensis*. Accordingly, they were set out in a block far removed from other *Cryptostegia* plants for further observation. The main characters of this hybrid have been described by Polhamus, Hill and Elder (6). They noted that the hybrid plants were marked by greater vigor but in other respects were intermediate between the two parents. Table I illustrates some of the more apparent differences noted by these workers together with observations made in the present study. Further indication that these

TABLE I

*A Comparison of Some Characters Which Serve to Distinguish the Two Species of Cryptostegia from the Intermediate Hybrid*

Character	<i>C. grandiflora</i>	<i>C. madagascariensis</i>	Hybrid
1. Habit of growth	Large, climbing shrub	Compact, bushy shrub	Larger and more vigorous than either parent. Tends to climb
2. Leaves	Narrow and thin with a purple midrib	Thick, glossy, elliptical; midrib not colored	Narrow and thin midrib not colored
3. Flowers	Pale purple	Purple	Light purple
4. Runner-like Whips	Present	Usually Absent	Present
5. Latex quality	High rubber, low acetone extract	High acetone extract, little or no rubber	Like <i>grandiflora</i>
6. Chromosome number	$n = 11$	$n = 11$	$n = 11$

plants were natural, interspecific hybrids was indicated by observations on segregation and recombination of some characters in the second generation progenies.

Attempts were made to cross the two species under controlled conditions of pollination. All efforts ended in failure regardless of which species was chosen as pollen parent and whether or not insect or hand-pollination was tried. Similarly, no fertilization occurred in hybrid ovaries pollinated with either parent, nor was there any conclusive evidence that such crosses occur naturally. The hybrid, however, produced abundant seed although it is not known whether this was the result of sibbing or self-fertilization.

*The F<sub>2</sub> Progenies.* The F<sub>2</sub> population consisted of six progenies, with an average of 33 plants in each, which were derived from seed produced by open-pollination of individual F<sub>1</sub> hybrids. Significant variations in latex quality and other characters such as flower color, leaf shape and habit of growth indicated that these progenies resulted from fertilization of hybrid plants.

The F<sub>2</sub> plants could be fairly easily grouped into classes resembling one or the other of the two parents or the hybrid. No distinctly new

morphological types were observed. Originally, 330  $F_2$  plants were planted as a block. Lack of cultural care, however, reduced the number to something less than 300 plants. A total of 168 plants has been analyzed for latex quality at some time during the progress of this work.

*Chromosome Numbers.* *Cryptostegia* chromosomes are very small and cytological work was limited to determinations of chromosome numbers. These were made on flower buds by the aceto-carmin anther smear method. In material examined, products of the two meiotic divisions appeared to remain together to form a pollen tetrad.

A haploid complement of 11 chromosomes was found for all plants examined. These included counts from 20 *C. madagascariensis* plants representative of the various stocks used in the present study. Flowers were examined from *C. grandiflora* plants growing in the states of Tamaulipas and Sinaloa, Mexico, and from two stocks at the Plant Introduction Garden, Cocoanut Grove, Florida. Seeds of one of these stocks came from Bombay Province, India, and the other from Brisbane, Australia. Six  $F_1$  hybrid plants also gave counts of 11 bivalents. The pairing of chromosomes was observed to be complete. The chromosome behavior of normal pairing in meiosis in the interspecific hybrid is in harmony with regular segregation and recombination of genetic factors.

*Collection of Latex.* As indicated in Table II, there was a distinct and completely reproducible difference in the latex quality of *C. madagascariensis*, depending on the age of the stem from which the

TABLE II

*Variation in Quality of Oven-Dried Latices from Different Parts of the Branching System of a Single Plant of C. madagascariensis*

Zone number	Part of plant sampled	Average diameter in mm.	Percentage	
			Acetone extract	Rubber
1	Trunk	65	8.1	68.5
2	Large branches	40	7.4	69.7
3	Medium-sized branches	24	10.0	72.7
4	Medium-sized branches	15	23.5	54.2
5	Smaller-sized branches	10	61.1	14.2
6	Young shoots	3-4	72.8	1.6

latex was removed. As stems increased in size, more rubber was found in the latex. This was presumably the result of a difference in the quality of pith and cortical latex, the former containing a much larger amount of acetone extract than the latter. In sampling young stems of either *C. madagascariensis*, *C. grandiflora*, the hybrid or the  $F_2$  plants, it was observed that most of the latex was derived from the pith. With larger stems, two distinct and almost equal regions of flow were observed, while in larger trunks, it was apparent that most of the latex originated from the cortex. Every effort was made to secure latex from young tissue comparable in age and size.

To secure latex from *C. madagascariensis*, small branches were cut off approximately between the ninth and tenth internode. For purposes of internode designation, the youngest internode having a length of at least 1 inch was considered as number 1 and the count was made toward the point of union of the branch and next larger stem. Latex samples were obtained in a similar fashion from the elongated "whips" of *C. grandiflora* and the hybrid, although these plants were found to have the same latex quality irrespective of whether the latex was secured predominantly from the pith or cortex.  $F_2$  plants were bled in a similar manner.

Latex collections were made while the plants were dropping leaves (February 1944), near the end of the growing season, shortly before leaf-fall, (October 1943), and during the summer when growth was at a maximum (July-August 1944). Before the last sampling was made, all of the  $F_2$  plants were drastically pruned so that it was possible to examine latex from new tissue strictly comparable in age and size. Generally, enough latex would bleed from one cut for analytical purposes. In a few instances, two cuts were required.

*Analytical Methods.* Latex samples were either dried by heating to about 60°C. or were coagulated by adding one fourth volume of acetone and then cooling for two hours in a refrigerator. The coagulated sheets were washed with water and pressed repeatedly after which they were dried over  $P_2O_5$  at room temperature. A weighed aliquot, about 0.150 g. was finely divided and then extracted with redistilled acetone for 16 hours in a Bailey-Walker extraction apparatus. The acetone-soluble materials were regained by evaporation of the solvent at 50°C. in a stream of air and weighed.

Rubber hydrocarbon was determined either by benzene extraction (Bailey-Walker) or by a slight modification of the iodination method proposed by Kemp and Mueller (7). The acetone-extracted samples were transferred to 250 ml. glass-stoppered flasks, 30 ml. of  $CS_2$  were added and the samples allowed to swell in this medium for 16 hours. Twenty-five ml. of *Wijs* solution, (ICl), prepared and preserved according to the directions of Kemp and Mueller, were added and the flasks and their contents placed in the dark for one hour. Fifteen ml. of a 15% solution of KI were added together with 50 ml. of freshly boiled and cooled distilled water. The excess iodine was titrated with 0.1 *N* sodium thiosulfate, using starch as an indicator. The degree of unsaturation, hence *per cent* rubber, was calculated after correction for a blank.

Analyses for the hot acetone-soluble triterpene ester, whose properties have been described, (5) were based upon its relatively low solubility in cold acetone. The

acetone extract was redissolved in boiling acetone and quantitatively transferred to tared centrifuge tubes which were then placed in an oven at 50°C. to reduce the volume to 3 ml. The tubes and their contents were next placed in a refrigerator at 4°C. for 16 hours to allow the ester to crystallize, after which they were centrifuged for several minutes in the refrigerator. The supernatant fluid was decanted and the precipitated ester was quickly washed with 3 to 4 ml. of ice-cold acetone which was decanted after centrifuging. The crystallized ester was dried over  $\text{CaCl}_2$  *in vacuo* for 5 hours, and then weighed.

The "true" amount of ester in a coagulated latex was given by the sum of the observed weight and the amount remaining in solution in 3 ml. of acetone at 4°C. The solubility of the ester under these conditions was found to be 12 mg. in 3 ml., or approximately 8% of the sample weights used. This correction introduces uncertainty in ester values which are below 10%. Small precipitates, such as found in *C. grandiflora* coagula, were not definitely identified as ester, and all that can safely be stated in such cases is that the ester does not exceed 10% of the sample and well might be 0%.

### EXPERIMENTAL RESULTS

During February 1944, latex samples were bled from 72  $F_2$  plants and analyzed for total acetone extract and rubber. The results are presented graphically in Fig. 1. Previous analyses had shown that *C.*

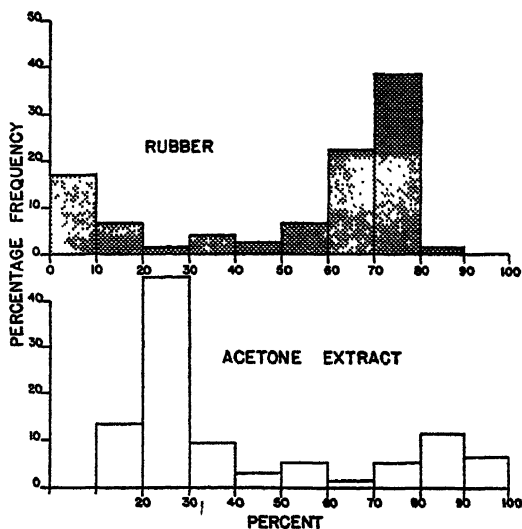


FIG. 1

Acetone Extract and Rubber Contents of 72  $F_2$  Plants from  
*C. grandiflora*  $\times$  *C. madagascariensis*

*grandiflora* latex always contained less than 20% acetone extract and 70–80% rubber, while *C. madagascariensis* latex contained more than 80% acetone extract and less than 5% rubber. The natural hybrids closely resembled *C. grandiflora* in having 25–35% acetone extract and 60–65% rubber, although there was a consistent tendency for the hybrid to have slightly less rubber and slightly more acetone extract than *C. grandiflora*.

It is evident from Fig. 1 that segregation for latex quality had occurred in the  $F_2$  generation, with rubber being the predominant substance synthesized in most of the latices. The plants at this time were in a semi-dormant condition and had not been disturbed for more than 10 years. There was no apparent correlation between the obvious morphological characters of the individual plants and the quality of the young shoot latex, although plants having the morphological characteristics of the parental types appeared to have segregated. Evidently, independent segregation for latex quality had also occurred as about 75% of the plants could be classified as having latex containing large amounts of rubber and little acetone extract while the rest were low in rubber and high in acetone extract.

When it became evident that lupeol ester was a principal constituent of *C. madagascariensis* latex, the  $F_2$  plants were again examined in July–August 1944 for latex quality together with the  $F_1$  hybrid and the two parental types. Latex was analyzed from 126  $F_2$  plants. Table III illustrates the latex quality encountered in the parental types and the hybrids. It can be observed that both parental types and the hybrid fall into distinctly different, narrow percentage ranges with respect to rubber, acetone extract and lupeol ester. It is equally clear that the hybrid more nearly resembles *C. grandiflora* in latex quality than *C. madagascariensis*.

Since the  $F_2$  plants were in a very over-grown condition, all the plants were drastically pruned back to a few main stems in March 1944, to eliminate crowding as an environmental factor which might have affected latex quality. Enough new growth occurred by July–August 1944 so that sufficient latex could be collected from individual plants for chemical analysis from new tissue strictly comparable in age and size.

Table IV is a histogram of the total acetone extract obtained from precipitated coagula. The graph is arranged so that actual plant



TABLE III

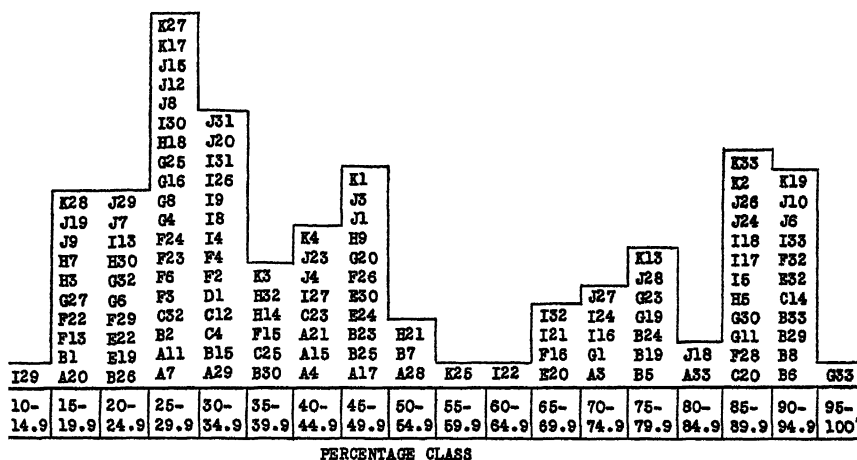
*Acetone Extract, Ester and Rubber Percentages of Coagula Obtained from Stem-Latex of C. grandiflora, C. madagascariensis and their F<sub>1</sub> Hybrid*

	Plant numbers	Percentage		
		Acetone extract	Ester	Rubber
<i>C. grandiflora</i>	7	11.5	10% blank	73.1
	8	14.1	blank	73.0
	9	12.0	blank	73.3
	10	11.4	blank	74.6
	11	12.1	blank	73.7
	12	13.3	blank	72.2
	13	14.0	blank	70.0
	14	12.8	blank	73.1
	Mean	12.6	blank	72.9
	S.E.M.	0.38	blank	0.46
	C.V.	8.4	blank	1.8
<i>C. madagascariensis</i>	1	87.0	52.4	3.0
	2	86.0	54.1	2.3
	3	85.5	49.8	1.8
	4	88.9	54.3	2.8
	5	89.2	55.6	2.2
	6	86.6	54.2	3.0
	Mean	87.2	53.4	2.5
	S.E.M.	0.60	0.83	0.20
	C.V.	1.7	3.8	18.9
<i>F<sub>1</sub> (C. grandiflora × C. madagascariensis)</i>	B8	28.5	16.3	60.1
	C1	33.7	15.6	61.2
	C13	29.9	15.2	60.1
	D10	29.5	14.0	63.0
	E4	33.8	17.1	59.3
	E9	33.2	15.6	61.6
	F6	30.5	14.3	62.7
	F17	29.5	13.9	62.2
	I17	30.8	14.9	60.5
	Mean	31.1	15.2	61.2
	S.E.M.	0.67	0.36	0.41
	C.V.	6.4	7.1	2.0

numbers are placed in their respective percentage classes and thereby emphasizes the purely random spread encountered in latex quality among the individual  $F_2$  plants. "Place effect" also appears to be eliminated from consideration since the block was planted so that rows running South to North were designated by the successive letters, A, B, . . . K, while the rows running West to East were designated

TABLE IV

*A Histogram of Individual  $F_2$  Plants Classified on the Basis of Acetone Extract in the Latex Coagulum*



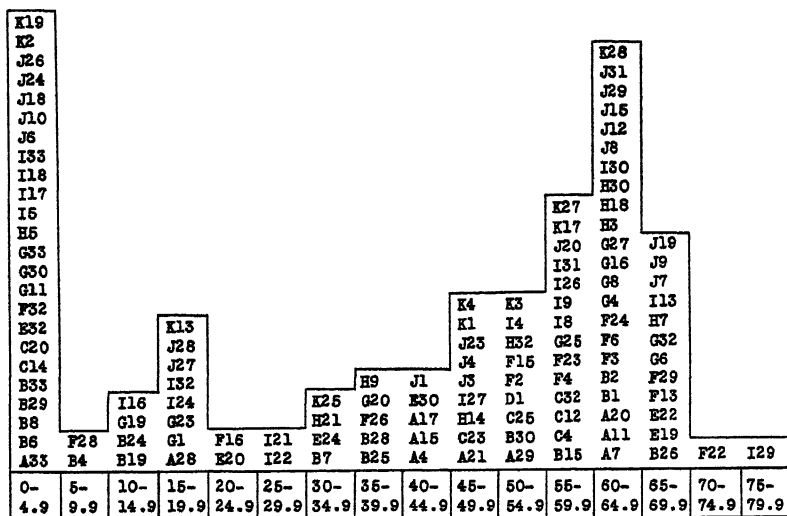
by the successive numbers, 1, 2, . . . 33. Each progeny obtained from  $F_1$  seed, of which there were six, was planted in order. Thus, the  $F_2$  plants obtained from hybrid plant A1 were planted as E18, E19 . . . E33, F1, F2, . . . F33, G1, G2 . . . G11. Almost one-half of the total number of  $F_2$  plants were examined in this experiment.

Table V is a histogram arranged in a similar manner to illustrate segregation for rubber. Most of the plants are like *C. grandiflora* in containing rubber as the dominant constituent of their latices while the rest are either intermediate or like *C. madagascariensis*.

Table VI is a histogram showing that high-ester types apparently segregate in a recessive fashion. Fig. 2 is a graph which shows that an inverse correlation exists between rubber and lupeol-ester formation. With high ester values little rubber is formed, and *vice versa*.

TABLE V

### A Histogram of Individual $F_2$ Plants Classified on the Basis of Rubber Content in the Latex Coagulum



PERCENTAGE CLASS

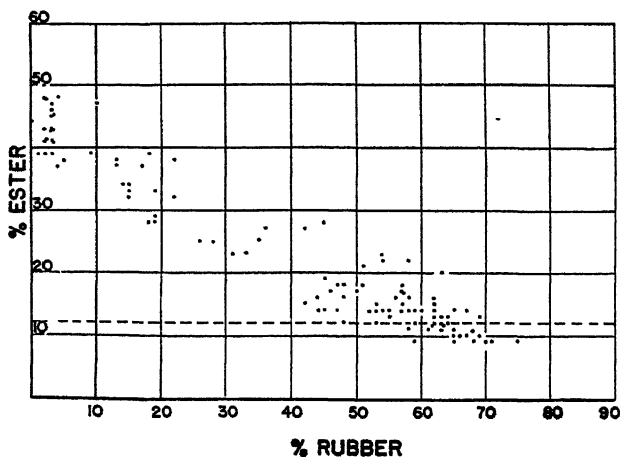


FIG. 2

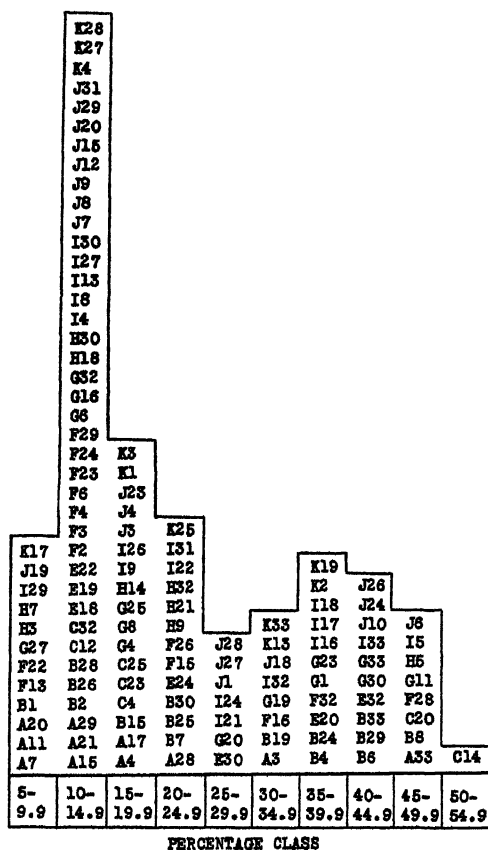
### Variation of Lupeol Ester and Rubber Contents in F<sub>2</sub> Plants from *C. grandiflora* × *C. madagascariensis*

## DISCUSSION

Examination of the acetone and rubber histograms shows the presence of two major divisions of latex quality and indications of minor divisions in the frequencies of the intermediate classes. The lupeol-ester histogram shows this same tendency although the minor peaks are not as clearly indicated, probably due to the large experimental error introduced in the analytical determinations for ester.

TABLE VI

*A Histogram of Individual F<sub>2</sub> Plants Classified on the Basis of  
Lupcol-Ester in the Latex Coagulum*



These data are in agreement with the concept that a dominant gene (or genes) controls the enzymatic formation of rubber by which a simple precursor is condensed into a linear polymer of high molecular weight. Similarly, a gene (or genes) whose expression is recessive to that of rubber formation controls the enzymatic formation of lupeol ester by cyclic condensation from the same substrate. The information, however, is not sufficiently extensive to show that the genes are alleles, since the expression of such genes may have been modified in the  $F_1$  and  $F_2$  by competition of other, unknown mechanisms, utilizing the same substrate, accounting for the portion of the total acetone extract that was not lupeol ester.

Approximately 60% of the acetone extract obtained from *C. madagascariensis* latex is a triterpenol esterified with a mixture of long-chain, hydroxy fatty acids (5). Triterpenes are now rather well known to be pentacyclic hydrocarbons, although the structures are not completely worked out. The structure of lupeol, as suggested by Ruzicka and Rosenkranz (8), is given in Fig. 3. In this figure one can see separate isoprene-type structural elements that are considered to be preserved in this type of compound. Rubber, the formula of which is also given in Fig. 3, is a linear polymer of isoprene-type structural elements. The double bonds appearing in rubber are considered to be equivalently expressed by the cyclization of the triterpenol. In short, rubber is an extended linear polymer while triterpenes are limited cyclic polymers that could well be formed from the same precursor.

Latex systems are discrete, homogeneous tissues confined to a small number of plant families, some of which are rather widely separated phylogenetically. Latex tubes are probably restricted in their metabolic activities—as far as the coagulum is concerned—to the production of relatively few major compounds most of which are structurally related. Ratios of amounts of these compounds forming under widely varying environmental conditions are quite constant. In *Cryptostegia*, the parental species, the  $F_1$  hybrid and  $F_2$  progenies have been sampled at different seasons of the year representative of varying metabolic conditions. No essential difference in the ratio of acetone extract to rubber was detected which could be referred to a seasonal effect. It was also found that the significant correlation between rubber and ester was independent of variations (6–23%) in the amount of coagulum formed in individual latices.

In the differentiation of the two morphologically distinct species of *Cryptostegia*, it is probable that genes responsible for the production of the major constituents of the coagulum have remained relatively unchanged, as might be expected if they regulate basically related chemical processes. Several facts support this hypothesis: (1) The uniform occurrence of either rubber or lupeol ester in the two species

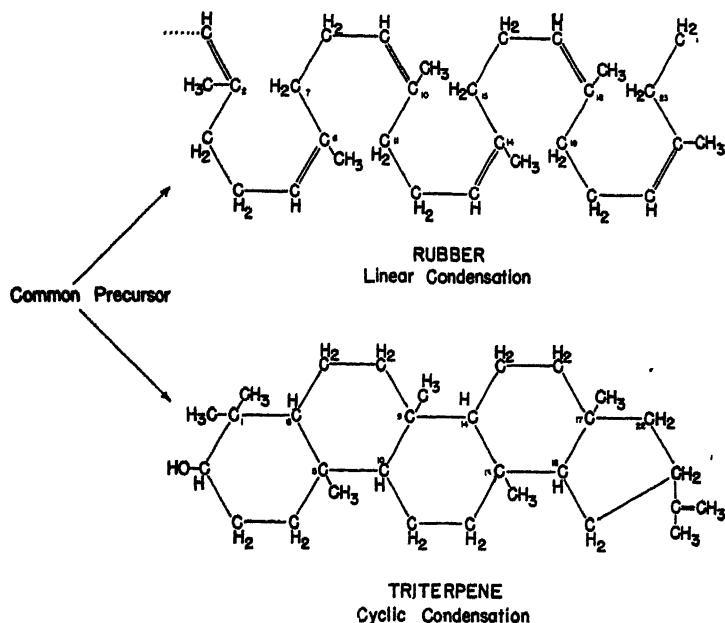


FIG. 3

Formulas of Rubber and Lupeol Indicating a Relationship of Linear and Cyclic Condensation

and the apparent segregation of the same compounds in the  $F_2$  generation. (2) The striking similarity in the segregating products, rubber and ester. A relatively minor change in the action of a gene or genes may be all that is necessary to change the direction of polymerization from rubber to ester.

Many other latices contain triterpenes. Lupeol, either free or esterified with acetic, isovaleric, caproic or cinnamic acids, has been found associated with rubber in species of *Alstonia*, *Ficus*, *Phyllanthus*,

*Butyrospermum*, *Plumeria*, *Dyera* and *Cryptostegia*. With gutta, it has been found in *Achras*, *Palaquium* and *Mimusops*. (9) Similarly, the triterpenes  $\alpha$ - and  $\beta$ -amyrin are widely distributed in latices. The list could be extended.

It is doubtful if any latex has been examined in which the predominant constituents in the non-aqueous phase of the latex emulsion have not been confined to isoprene-type hydrocarbons. The most satisfactory hypothesis to account for this phenomenon is that a relatively small number of genes determine the direction for polymerization of isoprene substances and, irrespective of speciation, no great change has occurred in the restricted function of such genes as manifested by the chemical products they control.

We are indebted to Mr. Loren G. Polhamus of this Bureau and to Dr. James Bonner and Dr. Sterling Emerson of the California Institute of Technology for stimulating discussions and helpful criticisms.

#### SUMMARY

1. Young shoot latex obtained from *Cryptostegia grandiflora*, *C. madagascariensis*, a natural hybrid, *C. grandiflora*  $\times$  *C. madagascariensis* and an  $F_2$  population derived from the hybrid was examined for latex quality.

2. *C. grandiflora* latex coagulum uniformly contained less than 20% acetone extract and 70–80% rubber. *C. madagascariensis* latex coagulum contained less than 5% rubber and more than 80% acetone extract of which more than 60% was accounted for as a triterpene (lupeol) ester. The hybrid resembled *C. grandiflora* in latex quality.

3. In the  $F_2$  generation, approximately 75% of the plants contained predominant rubber, while approximately 25% contained predominant ester in the latex coagulum.

4. Evidently, segregation for latex quality occurred but the data were not sufficiently extensive to show that rubber and lupeol ester formation was controlled by allelic genes, although the experimental data suggest such a scheme.

5. It is concluded that a common precursor is utilized in the formation of rubber and lupeol ester. General evidence for a common precursor is afforded by the association of rubber or gutta with triterpenols in the latex systems of many other plant species.

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# A Triterpene Ester Isolated from *Cryptostegia madagascariensis* Latex

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## INTRODUCTION

During the course of an investigation on the inheritance of latex quality in *Cryptostegia*, the following facts were established (1):

1. The coagulum obtained from *C. grandiflora* latex uniformly contained 70-80% rubber and less than 20% acetone extract.
2. Conversely, *C. madagascariensis* latex coagulum obtained from small branches contained less than 5% rubber and as much as 90% acetone extract.
3. The natural hybrid formed from these two species more nearly resembled *C. grandiflora* in latex quality than *C. madagascariensis*.
4. In the F<sub>2</sub> population derived from the hybrid, approximately 25% of the plants contained predominant ester while 75% contained predominant rubber in the latex coagulum.

In view of the segregation of latex quality in the F<sub>2</sub> generation, it was of interest to examine the latex of *C. madagascariensis* from a chemical standpoint. The following work shows that the principal constituent in the coagulum of this latex is a triterpene-ester. The hypothesis has been presented (1) that the formation of rubber or ester in *Cryptostegia* latex is controlled either by a single gene or by a few genes, and that both polymers are derived from a simple, common precursor.

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## DISCUSSION

Coagulum derived from latex of young branches or vegetative shoots of *C. madagascariensis* is almost completely soluble in hot acetone and a single substance, amounting to more than half of the starting material, separates upon cooling. This substance (I), with an empirical composition near  $C_{53}H_{94}O_3$ , is a mixture of esters of a triterpenol.

Properties of the triterpenol (II) obtained by saponification of the ester mixture (I) shows it to be identical with lupeol. It is similar in properties, as shown below, to  $\beta$ -calotropeol, the acetic and isovaleric esters of which are the principal latex constituents of *Calotropis gigantea* (L.) R. Br. (2). Identity of the triterpenol (II) and lupeol was completely established by the crystallographic constant of the derived ketone and the previously reported values for crystals of lupenone (4).

TABLE I

Property	Triterpenol (II)	Lupeol (3,4)	$\beta$ -Calotropeol
Composition	$C_{50}H_{98}OH$	$C_{50}H_{98}OH$	$C_{50}H_{98}OH$
Melting point, m.p.	216–217°	215°	216–217°
$[\alpha]_D^{25}C_6H_6$	+33°	—	+51°
Acetate, m.p.	217–218°	214–215	238°
Acetate, $[\alpha]_D^{25}CHCl_3$	+42.5	+47.3	—
Benzoate, m.p.	260°	265°	279–280°
Ketone, m.p.	170.5–171.2	169.5–170.5	—
Ketone, $[\alpha]_D^{25}CHCl_3$	+58.5°	+63.5°	—

About two-thirds, 70%, of the mixed acid portion of the ester (I) proved to be a mixture of 2-hydroxy-*n*-fatty acids as shown by chemical analyses, formation of insoluble Mg salts in 95% ethanol (5, 6) and oxidation, with a loss of one C atom/molecule, to normal fatty acids. That oxidized acids are normal is shown by the properties of their ethyl esters and by presence of (h k 0) X-ray diffraction maxima characteristic of normal fatty acids. The 2-hydroxy-*n*-fatty acid mixture is a complex one and possibly includes components between  $C_{16}$  and  $C_{26}$  acids with maxima occurring near  $C_{18}$  and  $C_{24}$  or  $C_{17}$  and  $C_{23}$  with the higher carbon fraction predominant. None of the fractions separated contained acids of sufficient purity to permit safe deductions as to whether or not only acids with even numbers of carbon atoms are present. This fraction is similar in part to "phrenosinic acid," a

component of the cerebrosides phrenosin and kerasin (5), which has been shown to be a mixture of 2-hydroxy-*n*-docosanoic, 2-hydroxy-*n*-tetracosanoic and 2-hydroxy-*n*-hexacosanoic acids (6), that can be oxidized easily to the acid mixture commonly known as "lignoceric acid."

The remainder of the acid moiety (30%) formed Mg salts which were very soluble in 95% ethanol. The fraction was not completely characterized. Complexities of naturally occurring acid mixtures of the type encountered here have been emphasized by the work of Chibnall and his associates (7) as well as by the recent work of Weitkamp on wool scourings (8).

### CHEMICAL EXPERIMENTS

*Fractionation of Latex.* Latex was obtained from young stems and vegetative shoots of *C. madagascariensis* at Coconut Grove, Fla., and shipped to Washington, D. C. Three days after collection, 197 g. of this latex were coagulated by adding 100 ml. of acetone with thorough stirring. After 1.5 hours complete coagulation was apparent and the clear serum was removed by filtration. The coarsely granular white precipitate was repeatedly washed with water and then vacuum dried at room temperature. The yield was 52.8 g. or 26% of the total latex. 600 g. of a 2nd lot of *C. madagascariensis* latex from pods gave 150 g. or 25% of coagulum and the serum contained 39 g. of solids (6.5%), 33.4 g. (5.6%) of which were soluble in absolute methanol and insoluble in benzene.

The coagulum obtained from young branch latex was extracted with acetone in a soxhlet. A small insoluble residue (1.0 g.) was extracted with petroleum ether and the solution was evaporated giving 0.5 g. of rubber (0.25%).

The combined acetone solutions were reduced in volume and then cooled to 4°C. A white crystalline precipitate (I) separated (60% of coagulum). The cold acetone washings and mother liquor, evaporated to dryness, gave a highly viscous material from which characterizable compounds were not obtained.

*Purification of Fraction Insoluble in Cold Acetone (I).* Four recrystallizations from acetone raised the m.p. from 72°-75° to 76.0°. It was not changed by two further crystallizations from acetone and one from diethyl ether. Other constants of the material are:  $[\alpha]_D^{25}$  benzene = + 16.1°; m.w., Micro-Rast (camphor) 807,793, by saponification (as a single ester) 772, by addition of IBr (Hanus) 785 (for one double

bond), no addition of  $\text{Br}_2$  in  $\text{CCl}_4$ ; Sol. 4.3 mg./ml. at  $4^\circ\text{C}$ . in acetone, moderately soluble in methanol, ethanol, acetone and ether, extremely soluble in nonpolar solvents.

Anal.; Calc.,  $\text{C}_{23}\text{H}_{31}\text{O}_3$  (m.w. 377.7), C, 81.68, H, 12.15.

$\text{C}_{24}\text{H}_{33}\text{O}_3$  (m.w. 391.7), C, 81.74, H, 12.20.

Found, C, 81.4, 81.4, H, 11.86, 11.81.

*Saponification of Mixed Ester (I).* The procedure outlined by Chibnall *et al.* (9) was generally followed. Yields of crude alcohol (II) and acid (III) regenerated from the Ca salt were 97%, 95% (II) and 85%, 98% (III), respectively, in two experiments.

*Triterpenol (II) = Lupeol.* The crude alcohol was crystallized four times from acetone and finally from petroleum ether. Loss in weight was 50% and the m.p.,  $216\text{--}217^\circ$ , did not change during the last three crystallizations. Other constants of the compound are:  $[\alpha]_{\text{D}}^{25}$  benzene =  $+33.0^\circ$ ; m.w., Micro-Rast (camphor), 430; color tests, Lieberman-Burchard, intense red, Salkowski, bright orange.

Anal.; Calc.,  $\text{C}_{30}\text{H}_{50}\text{O}$  (m.w. 426.7), C, 84.44, H, 11.81.

Found, C, 84.4, 84.6, H, 11.8, 11.8.

*Triterpenol (II) Lupeol Acetate* was prepared from acetic anhydride and fused sodium acetate. Its melting point after recrystallization from ethanol was  $217^\circ$ . The mixed melting point with (I) was depressed  $40^\circ\text{C}$ . Other constants of the compound are:  $[\alpha]_{\text{D}}^{25}\text{CHCl}_3$  =  $+42.5^\circ$ ; m.w. by saponification 456.

Anal.; Calc.,  $\text{C}_{32}\text{H}_{52}\text{O}_2$  (m.w. 468.7), C, 81.99, H, 11.18.

Found, C, 82.2, H, 11.30.

*Triterpenol (II) Lupeol Benzoate* was prepared by use of benzoyl chloride in the presence of a few drops of pyridine. It was crystallized to a constant melting point of  $260^\circ\text{C}$ . from ethanol.

Anal.; Calc.,  $\text{C}_{37}\text{H}_{54}\text{O}_2$ , C, 83.72, H, 10.25.

Found, C, 83.3, 83.4, H, 10.40, 10.55.

*Triterpenone, Lupenone* was prepared by oxidation of (II) with chromic acid at  $30^\circ\text{C}$ . (3). The yield was quantitative and twice recrystallized material (95% ethanol – 60% yield) melted at  $170.5\text{--}171.2^\circ$ ;  $[\alpha]_{\text{D}}^{25}$  in  $\text{CHCl}_3$  was  $58.5^\circ$ . Crystals were optically positive with a small 2V and strong dispersion with  $\rho > \nu$ . The crystal class is  $D_2 = 222$ , orthorhombic enantiomorphic hemihedral, as required for an optically active orthorhombic compound and not orthorhombic bipyramidal as reported by Jaeger (3) and realized by him to be doubtful.

*Mixed Acids (III).* 5.6 g. of the crude acid regenerated from the calcium salt was dissolved in 50 cc. of boiling 95% ethanol and to this was added 100 cc. of a 5% solution of magnesium acetate. The solution was filtered while hot to remove the insoluble magnesium salts of the  $\alpha$ -hydroxy fatty acids (5, 6). The free acids were regenerated by dissolving the magnesium salts in glacial acetic acid and then diluting with water. After cooling to  $0^\circ\text{C}$ . the acids were separated by filtration and washed until free of acetic acid. The yield of 2-hydroxy acids (III A) was 70%.

The filtrate from the above treatment was evaporated to dryness, taken up with glacial acetic acid and then diluted with water. The precipitated acids were redissolved in 95% ethanol and a second treatment with magnesium acetate was carried out. No precipitate formed and the free acid was again regenerated. The acid yield

(III B) was 30% of the crude acid. Analytical evidence was obtained indicating that III B was a mixture of long chain fatty acids and hydroxy fatty acids.

*2-Hydroxy-n-fatty Acids (III A).* The crude acid was recrystallized four times from acetone and three times from absolute methanol. The acid as purified melted at 84.8–85.2°C. and set sharply at 84.0°C. with a heating rate of about 1°C./minute. Other constants of this material are:  $[\alpha]_D^{25}$  in methanol = + 3° (inactive, within experimental limits); neutralization equivalent 372, 371; X-ray diffraction, (h k 0) reflections different from normal fatty acids were observed. (00l) reflections were absent.

Anal.; Calc.,  $C_{23}H_{46}O_2$  (m.w. 370.6), C, 74.54, H, 12.50.

Found, C, 74.4, 74.5, H, 12.32, 12.35.

These observations are typical of long chain fatty acid mixtures and are inadequate to serve as criteria for purity or evidence of straight- or branched-chain character (9).

In order to demonstrate heterogeneity of (III A) the "purified" acid was subjected to a triangular crystallization scheme with acetone as a solvent. Five *per cent* was obtained in the 4th step with a m.p. of 91.5°C. and a setting point of 90°C., which is about 6°C. higher than that of the starting material. It did not give (00l) X-ray diffraction maxima, which indicates inhomogeneity.

Anal.; Calc.,  $C_{23}H_{46}O_2$ , C, 74.54, H, 12.50,  $C_{25}H_{50}O_2$ , C, 75.33, H, 12.64.

Found, C, 75.47, H, 12.21.

*Oxidative Degradation of 2-Hydroxy-n-fatty Acids (III A).* The acid was oxidized with  $KMnO_4$  in acetone by the procedure of Levene and Taylor (10). Reaction was rapid and after two recrystallizations from dry petroleum ether a product was obtained melting between 62.4 and 62.8°C. with a setting point between 61°C. and 60°C. A depressed melting point and poor diffraction pattern are typical for normal fatty acid mixture containing several constituents. Other constants of this material are: Neutralization equiv. 345; X-ray diffraction, diffuse 1st and 3rd orders from (00l) gave a spacing of  $53 \pm 2 \text{ \AA}$  and (h k 0) reflections of normal fatty acids, required for  $C_{23}H_{46}O_2 = 51.8 \text{ \AA}$  (C modification, m.p. 79.6°C. (11) and  $C_{21}H_{42}O_2 = 47.8 \text{ \AA}$ , m.p. 75.2°C. (11).

Anal.; Calc.,  $C_{23}H_{46}O_2$  (m.w., 354.6), C, 77.83, H, 13.08.

$C_{21}H_{42}O_2$  (m.w., 326.6), C, 77.24, H, 12.96.

Found, C, 77.05, 77.30, H, 13.03, 13.15.

A second lot of the oxidized acid gave 5 fractions upon successive crystallizations from acetone, namely: (1) insoluble (K salt of acid); 8%, (2) m.p. 67–73°C.; s.p., 71.5°C.; some ash; 4%, (3) m.p. 62.5–63.5°C.; s.p., 62°C.; no ash; 6%, (4) cold acetone insoluble; m.p. 59–60°C.; s.p., 58.5°C.; 35%, (5) very soluble in cold acetone, m.p. 53–54°C.; s.p., 52.5°C.; 43%.

*Ethyl Esters of Normal Fatty Acids.* Ethyl esters were prepared of fractions (4) and (5), above, by refluxing with 5% HCl in absolute ethanol. The ethyl ester of fraction (4), after repeated recrystallization from absolute ethanol, had a m.p. of  $45.0 \pm 0.1^\circ$  and a setting point (s.p.) of  $43.8 \pm 0.1^\circ$ . The spacing of (00l) measured on four observed orders is  $32.7 \pm 0.3 \text{ \AA}$ . The value observed for an equimolar mixture of  $C_{20}H_{41}COOC_2H_5$  and  $C_{22}H_{41}COOC_2H_5$  by Francis, Piper and Malkin (11) is  $34.0 \text{ \AA}$  while a similar mixture of  $C_{18}H_{37}COOC_2H_5$  and  $C_{22}H_{41}COOC_2H_5$  has a (00l) spacing of  $32.5 \text{ \AA}$ . The melting point corresponds to that of  $C_{20}H_{41}COOC_2H_5$ , but is depressed due to mixing of compounds.

The ethyl ester of fraction (5) melted at 17°–18°C. after removal of some higher melting ester. The equivalent weight by saponification was 303. Corresponding values for  $C_{17}H_{35}COOC_2H_5$  and  $C_{18}H_{37}COOC_2H_5$  are: m.w. 312.5; m.p. 34.0°C. and m.w. 284.5; m.p. 25°C., respectively.

Francis, Piper and Malkin (11) determined the melting point for an equimolecular mixture of  $C_{16}H_{32}O_2$  and  $C_{18}H_{36}O_2$  acids to be 57.0°C.

*Micro Analyses.* Some of the micro analyses reported in this work were made by Mr. C. H. Van Etten of the Northern Regional Research Laboratories through the courtesy of Dr. Reid T. Milner and others by Dr. Gertrude Oppenheimer of the California Institute of Technology through the courtesy of Dr. A. J. Haagen-Smit.

### SUMMARY

Lupeol esterified with a mixture of fatty acids is a major constituent of latex from *Cryptostegia madagascariensis*. Normal  $\alpha$ -hydroxy fatty acids make up about 70% of the mixture. Lupeol apparently is derived from a precursor of rubber in the latex.

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## Note on Vitamins in Fungi

The occurrence of some of the water-soluble, growth-promoting vitamins in fungi has been extensively investigated but the presence of the fat soluble vitamins has received little attention. In the current studies, determinations were made for the occurrence of the fat-soluble vitamins A, D and E, and also the water-soluble vitamin C. Assays were made of six fungi: *Alternaria solani*, *Thielavia terricola*, *Rhizoctonia solani*, *Helminthosporium sativum*, *Fusarium moniliforme*, and *Fusarium oxysporum* f. *lycopersici*. The fungi were grown in Richard's solution and the mycelia were obtained by filtering the media through cheese cloth. Excess moisture was removed by blotting the mycelial mat with filter paper. Approximately 0.5 g. of fungus tissue was ground with powdered glass for each assay and specific methods of extraction for each analysis were carried out as described.

Vitamin C determinations were made as described by Loeffler and Ponting (3) with the modification that a 0.3% solution of oxalic acid was used in the extraction. No reduction of dichlorophenol-indophenol was observed with any of the fungus extracts or with the media in which they were cultured.

Vitamin A was determined by the Moore-Davis (5) procedure and vitamin D by the Brockmann and Chen (1) method. Vitamin E was assayed by two methods, the Emmerie and Engel (2) ferric chloride-dipyridyl, and the Meunier and Vinet (4) Prussian blue techniques. Extracts of pig liver and of *Oleum Percomorphum* were included in the vitamin A and D assays as checks on the methods. No vitamin A, carotene, or vitamin D was found in any of the fungi as indicated by the absence of the proper color changes with antimony trichloride. A deep violet color was obtained with the extracts of pig liver and the fish oil concentrate.

Results obtained in the analysis for vitamin E were variable and depended on the method employed. The procedure of Emmerie and



Engel (2) showed the absence of the vitamin in all the fungi and its presence in wheat-germ oil and pig liver. It is based on the reducing properties of tocopherol toward ferric chloride and upon the color of ferrous salt in the presence of dipyridyl. With the Meunier and Vinet (4) procedure, positive tests were obtained for *Th. terricola* and *F. oxysporum* f. *lycopersici*. This procedure is, however, general for 1,2- or 1,4-diphenols and compounds of this type could be present in some fungi.

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University of Delaware,  
Newark, Delaware  
January 30, 1946

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## Pyolipic Acid, a Metabolic Product of *Pseudomonas Pyocyanea*, Active Against *Mycobacterium Tuberculosis*

In a recent report Davide (1) described the bactericidal effects of cultures of *Ps. pyocyanea* and *B. proteus* on *M. tuberculosis* and reported therapeutic results in infected animals.

Chemical fractionation of the cultures of *Ps. pyocyanea* has yielded an acidic lipid, which we have named "pyolipic acid." This substance possesses most of the activity as determined by the procedure described by us (2).

Pyolipic acid was obtained by extraction of the bacterial cells with suitable solvents and fractionation of the crude lipoids by means of buffer extractions.

The active fraction was a colorless, odorless, viscous oil readily soluble in all lipid solvents. The lead salt was insoluble in water but soluble in alcohol or ether. The neutralization equivalent averaged 500. Using a synthetic medium more than 1 g. of pyolipic acid could be obtained from a liter of culture. Using the manometric technique it was found that 0.125 mg. of pyolipic acid inhibited the oxygen consumption of 3 mg. *M. tuberculosis* in 3.5 ml. of glycerine broth (dilution 1:30,000).

Acid or alkaline hydrolysis yielded about 75% of the material as ether-soluble acids while the remainder represented water-soluble products of carbohydrate nature. The acids were esterified with diazomethane and subjected to vacuum distillation. Over 80% of the material distilled at 97°C. @ 0.9 mm. Analysis and molecular weight determinations established the formula as  $C_{11}H_{22}O_3$ ,  $[\alpha]_D^{20} = -18.9^\circ$ . The presence of a hydroxyl group was demonstrated by the formation of a monoazoate, m.p. 43-44°.

Saponification yielded a hydroxyacid ( $C_{10}H_{20}O_3$ ) m.p. 46-47°; hydrazide m.p. 134-136°C. and S-benzylthiuronium salt m.p. 131-132°C.

When this hydroxydecanoic acid was treated with chromic acid at room temperature five atoms of oxygen were consumed per mole with the formation of *n*-octanoic (caprylic) acid and CO<sub>2</sub>. This finding thus proved that the main hydroxyacid of pyolipic acid is (–)  $\beta$ -hydroxy-*n*-decanoic acid.

This acid does not seem to have been isolated from natural sources before. Through the kindness of Dr. Stållberg-Stenhagen we obtained a sample of the synthetic *dl*- $\beta$ -hydroxydecanoic acid, m.p. 56°C.

This acid behaved in identically the same way on chromic acid oxidation and gave the same long X-ray spacing (3) as the natural acid.

In the crude fatty acid fraction resulting from the saponification of pyolipic acid, small amounts of the lower and higher homologues, *i.e.*,  $\beta$ -hydroxyoctanoic and dodecanoic acids, also seem to be present.

The aqueous phase remaining after ether extraction of the sulphuric acid hydrolysate did not reduce Fehling's solution but gave a red Molisch reaction.

After removal of the sulphuric acid with barium hydroxide, evaporation of the neutral solution yielded a hard light-brown glass.

Treatment of this material in dry pyridine with azoyl chloride gave two crystalline azoates melting at 276–277° and 232–240°C., respectively.

Further work on the characterization of these water-soluble split products is under way. A full report on the work reported here will be published in *Arkiv Kemi, Mineral. Geol.*

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April 22, 1946

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3. We are greatly indebted to Dr. Stenhagen, Upsala, for X-ray analysis.

## Book Reviews

**Hackh's Chemical Dictionary** (American and British Usage). Third Edition. Completely Revised and Edited by JULIUS GRANT, M.Sc., Ph.D., F.R.I.C. The Blakiston Company, Philadelphia 5, Pa. 1946. 925 pp. Illustrated.  $17 \times 25$  cm. \$8.50.

This new edition of Hackh's Chemical Dictionary is the first to appear since the death of Dr. Ingo W. D. Hackh, its originator, in 1938. It has been completely revised by Dr. Julius Grant, its present Editor, who was a collaborator with Dr. Hackh in the earlier editions. The number of pages has increased to 925 and the number of definitions to 57,000. There is no important departure from the objectives and scope of the earlier editions, except that the pronunciations have been omitted. Dr. Grant explains this omission on the ground that the pronunciations given have been of little use "since every chemist remains a law unto himself in such matters."

This capitulation may have been unavoidable as a choice between two evils, but it is unfortunate. One of the admirable features of this dictionary has been the evident desire of the editors to maintain uniformity of usage throughout the English speaking world and particularly as between Britain and America. Certainly differences in pronunciation are outstanding dissimilarities in the languages of these two countries and are very much in need of any possible unifying influence.

This edition, like the preceding ones, is as compendious as convenience and usefulness permit; it maintains skillfully the difficult balance between an adequate chemical dictionary and a chemical encyclopedia. Its definitions are clear, simple and concise, yet there is an astonishing amount of information in a small compass. The printing and the paper are excellent.

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**Dietotherapy, Clinical Application of Modern Nutrition.** Edited by MICHAEL G. WOHL, M.D., Associate Professor of Medicine, Temple University School of Medicine and Chairman of the Advisory Committee on Nutrition, Philadelphia Department of Public Health. W. B. Saunders Co., Philadelphia, Pa., 1945. xxii + 1029 pp. Price \$10.00.

This comprehensive volume, as the title suggests, has been compiled primarily for those studying or practicing medicine. As stated in the preface, "the science of nutrition has not in the past been part of medical discipline. . . . It is therefore hoped that the present book will provide the practicing physician and the student of medicine with a sound knowledge of both current advances in and practical applications of the rapidly expanding science of nutrition."

Fifty-eight contributors, most of them medical men, cooperated in this treatise. With so many authors the difficulties in obtaining congruity of style and interpretation are obvious. Dr. Wohl, as editor, has done an admirable job of coordinating the

material to give a smooth-reading, logical presentation. Very few typographical errors were noted in a careful reading of the book. Only in a few instances were contrary viewpoints expressed by different authors—*e.g.*, on page 155 appears the statement that vitamin D probably influences calcium metabolism through some channel other than absorption, whereas on page 310 one reads that “the most evident effect of vitamin D is to promote the absorption of calcium.”

As a whole, the various subjects are expertly handled. Only in one chapter, dealing with “Nutrition and the Athlete,” did the reviewer feel that the scientific standards fell down. Certainly the answers obtained from questionnaires sent to coaches and trainers of basketball teams cannot be regarded as constituting a sound scientific basis for devising the best diet for the athlete.

In the foreword to the book, Dr. Russell M. Wilder points out the widespread growing interest in nutrition. He then deals briefly with several somewhat controversial aspects of nutritional science, including dietary requirements, factors which condition malnutrition, criteria for diagnosing deficiency states, diet therapy, and the use of vitamin preparations in food enrichment and as concentrates. Although he stresses that these subjects demand much more research, he concludes with the statement that “it is inexcusable to fail to put to work immediately such knowledge as is now available and of proved worth.”

The book is divided into three main portions: I. Normal nutrition (21 chapters); II. Nutrition in periods of physiologic stress (5 chapters); III. Nutrition in disease (18 chapters). Most chapters are accompanied by an extensive, up-to-date bibliography.

The first section opens with a short outline of the functions of a normal diet and factors which influence the diet. Then, following a detailed description of the physiology of the gastrointestinal tract, the separate roles of water, carbohydrate, fat, protein, various mineral elements and the vitamins are discussed in logical order. Other chapters in this section which are of timely interest deal with the use of tracer (labeled) elements in nutrition studies, diagnostic methods for nutritional deficiencies, problems of nutrition in industry, and the influence of economic status and ignorance on the prevalence of malnutrition. Although the last two subjects deviate from the clinical approach evidenced in the preceding chapters, they are matters which definitely deserve more attention and cooperation on the part of the present-day physician.

Part II stresses the special nutritional needs during such periods of stress as pregnancy, childhood growth, old age, infection and elevated physical activity (athletics).

The third portion of the book deals primarily with the relation of nutrition to various pathological conditions. Included in this category are: dental disturbances, digestive diseases, diabetes mellitus, nephritis, urologic diseases, cardiovascular disease, allergies, Addison's disease, anemias, diseases of the nervous system and infectious diseases. Obesity and leanness and the nutrition of the psychiatric patient are also discussed. Other chapters are devoted to the nutritional care of the surgical patient, the special problems arising from gastro-intestinal surgery, and the new use of amino acids as protein nourishment.

In an appendix, the editor has brought together miscellaneous practical information for ready reference. Sample hospital diets and formulas for tube feedings are presented. Various methods for preserving and processing foods are briefly discussed, together with suggestions for conserving the nutrients in foods. Other subjects include: the composition and character of various foods (dairy products, meat, fish, cereals, fruits and vegetables, and beverages); a set of food charts showing the best dietary sources of certain vitamins, protein, calcium and iron; a table listing the nutrient composition of most commonly used foods; the approximate carbohydrate content of fruits and vegetables; suggestions for food substitutions; weight and capacity measures. An excellent report on the nutritional aspects of convalescent care, issued originally by the National Research Council's Committee on Convalescence and Rehabilitation, is reproduced in full.

The book contains 93 illustrations, numerous tables, and has a comprehensive subject index.

The reviewer believes that this volume will prove of particular interest and value to the medical student and practitioner and will serve as a useful, up-to-date reference work for specialists in related biological fields.

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**Die Ernährungsphysiologische Bedeutung der Hefe.** Beihefte zur Zeitschrift für Vitaminforschung Nr. 4. By Dr. J. C. SOMOGYI. Medizinischer Verlag Hans Huber, Bern. 110 pp.

This small volume is a factual and detailed review of the nutritional aspects of yeast and yeast extract. The author covers the literature to about 1942. There are a few references to work published in 1943.

It was the author's intention to summarize the data regarding the nutritional value and vitamin content of various types of yeast in order to encourage the use of yeast during normal times as well as during emergencies.

The second chapter deals with the histology and chemistry of yeast. Reference is made to recent work on serological differentiation of yeast and production of giant cells by means of chemical carcinogenic agents. The author includes some interesting photomicrographs with ultra violet illumination which claim to show a different internal structure of baker's, brewer's, and *Torula* yeast. A fairly thorough survey of the many substances which occur in yeast is presented. The occurrence of radioactive potassium and uranium and the several and varied nitrogen compounds is noted. S. fails to mention the unusual sulfur-containing sugar obtained from yeast by Japanese investigators. The vitamin content of yeast is treated at length with emphasis on the absence of Vitamin A, the variation of thiamine content, and the relatively constant content of riboflavin and nicotinic acid. The occurrence of ergosterol in yeast is also discussed. The author does not treat of yeast enzymes because he believes that almost all enzymes known at present occur in yeast.

In chapter III the obtention of brewer's yeast and the manufacture of wood sugar yeast and sulfite liquor yeast is discussed briefly. In the past the importance of yeast was due to its role in production of alcoholic beverages (baker's yeast is overlooked), but S. believes that its future lies in its great ability to synthesize substances of biological importance.

Chapter IV is a discussion of the methods for determining the nutritional value of proteins. The author is in accord with the view that the nutritional value of a protein depends on its content of essential amino acids but points out that questions of optimal proportion, availability, and the difficulties of amino acid determination, render the evaluation of a protein by analysis alone subject to great error. He does not mention or indicate that he is aware of the development in this country of microbiological methods for the determination of amino acids.

Chapter V deals with chemical investigations concerning the amino acids and proteins of yeast. The author stresses the limitations of the chemical approach as regards the nutritive value of yeast but agrees that certain helpful data can be obtained. He cites, as examples, investigations which show that yeast is low in histidine and tryptophane and high in lysine as compared to animal proteins. Much data on amino acid content are given and a comparison of the yeasts produced by different methods, *e.g.* wood sugar yeast, molasses yeast, sulfite liquor yeast, is presented.

Chapters VI and VII deal with biological determination of the nutritive value of yeast protein and with other feeding experiments using dried yeast. The important problem of the taste of yeast is discussed at this point. In Germany during the first World War the housewife was forced to use yeast, but its use was discontinued at the first opportunity. S. attributes this fact to the objectionable taste of yeast and describes extensive work done to improve it. Many workers obtained products which were claimed to be palatable but often mass production was impractical or the product was unacceptable for other reasons. In any event yeast has not found wide-spread use in the household.

The discussion of feeding experiments with dried yeasts is particularly critical and detailed. Experiments are described using sheep, swine, dogs and silver fox. The frequent errors of interpretation of the favorable effects of feeding a complex substance like yeast are discussed. He reviews at length unpublished experiments carried out in his laboratories on feeding yeast to growing dogs and hogs. He also studied the effect on the fat content of milk from cows fed yeast.

The author devotes a chapter (VIII) to the recent work done on increasing the thiamine content of yeasts other than brewer's yeast. Formerly brewer's yeast was usually slightly higher than other yeasts in thiamine content, but now *Torula* and baker's yeast can be produced to have a vastly superior content of this vitamin. This is accomplished by the addition of 2-methyl-4-amino-5-oxyethylpyridine and 4-methyl-5-oxyethylthiazole to the growing yeast. S. attributes this work to German investigators, overlooking the commercial use of this process in this country to produce a high thiamine yeast (Fleischmann's). Priority on this process should be given to Schultz, Atkin, and Frey (U. S. Patent No. 2,262,735 January 11, 1941).

The final chapter reviews investigations on yeast extract establishing that this substance is superior to meat extract both in quality and cost. The production, composition, and vitamin content are also considered. A practical method of distinguishing meat and yeast extract by means of ultraviolet spectroscopy is described.

The inclusion of information on the use of yeast in Europe, either as a human or stock food immediately prior to or during World War II, would have greatly enhanced the value of this book.

One merit of this contribution to yeast literature is that it discusses and reviews many publications relatively unavailable in this country.

Misspelling of proper names were the only errors noted. McCready is referred to as Cready, Heath as Heat, and C. A. Elvehjem as C. H. Elvehjem. The German is uncomplicated and the print and format are excellent. There are 301 references.

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**Biological Actions of Sex Hormones.** By HAROLD BURROWS, C.B.E., Ph.D., F.R.C.S. Cambridge, at the University Press; New York, the Macmillan Company. 1945. 514 pp. Price \$8.50.

The basic idea behind this comprehensive work was the preparation of a summary of present day knowledge of sexual endocrinology as it has developed from laboratory experimentation. The clinical side of the subject is not considered. The author expresses the hope that the treatise will serve as a "trustworthy . . . foundation for further progress in both sex-hormone research and clinical practice." This review of the status of the entire field of sex-hormone biology (through 1944) by one author is of unique interest in that a definite coherence is manifest in the presentation of the subject matter. This is a quality which is seldom attained by grouping of monographs by different authors, a common practice in recent years. The author has produced a very readable, well-documented assay. Considering the large mass of reference material (about 2000 citations), he is to be congratulated on the pertinent detail which he has woven into his summaries of experimental work. It is this characteristic which makes the book unusually valuable for reference purposes. Throughout the work the author does not hesitate to present his own views, although controversial topics are presented in an unbiased manner.

The book is divided into six parts; gonadotrophins, gonadal hormones, androgens, estrogens, progestins and the sex hormones of the adrenal cortex. Sex-hormone biology is a rapidly advancing subject and parts of any review of the subject will be out of date by the time it is printed. For example, recent isolation of several of the anterior pituitary hormones in pure form renders obsolete the detailed consideration of the question concerning the identity or non-identity of the follicle ripening and luteinizing hormones (pp. 12-17). However, this in no way detracts from the value of the book as a general reference work. The author's own investigations in the sex-hormone field were carried on, under the auspices of the British Empire Cancer Campaign, at the Chester Beatty Research Institute of the Royal Cancer Hospital. In his discussion of estrogens he has included a valuable chapter on the factors involved in the causation of mammary cancer and also considers in some detail the role of Bittner's milk factor.

The book is equipped with a detailed Table of Contents and a thorough index (28 pages). A special indexing of new growths is employed under the two headings of 'Cancer' and 'Tumours.' A complete alphabetical list of references is also provided. The printing is excellent on a good grade of paper and the book is remarkably free from printing errors.

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## Horse Brain Thromboplastin

### III. Stabilization of Activity of Suspensions\*

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#### INTRODUCTION

Thromboplastin suspensions of brain tissue that are used for the determination of prothrombin by the one-stage method of Quick (1) rapidly lose their activity on standing at room temperature, whereas at refrigerator temperatures this loss is retarded to some extent. Brain suspensions that were stable at refrigerator temperatures for various periods of time ranging from 1 day to 6 months have been recorded (2a, 3, 4, 5, 6, 7). Storage at room temperature is more detrimental although, in one instance (7), it has been reported that a saline suspension of rabbit brain remained active for 6 months. Solutions of purified lung thromboplastin have been stabilized with 1% sodium thiosulfate in sealed ampoules at room temperature for as long as 10 months; these thromboplastin solutions were oxygen-labile (8).

In our laboratory thromboplastin suspensions prepared from acetone-dried rabbit and horse brains proved to be quite unstable and only occasionally retained their useful range of activity for more than 1 day at room temperature and for only a few days at refrigerator temperatures. Since thromboplastin is widely used in clinical and

\* Some of the data in this paper were presented at the meetings of the Philadelphia Section of the American Chemical Society on July 13, 1945.

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research laboratories for the determination of prothrombin, it was felt that the preparation of a stabilized thromboplastin suspension would be of some interest and value.

Since dried brain powders gradually turn brown and lose their thromboplastic activity when exposed to air, presumably as a result of oxidation (2b), the possibility was considered that this same mechanism might also be responsible for the loss of activity of saline suspensions of brain powder. Accordingly, the effect of certain antioxidants on the stability of brain suspensions was investigated. However, subsequent observations suggested that bacterial contamination was partly, or perhaps entirely, responsible for this loss, and hence antiseptic substances were also studied. In this paper we wish to report the stabilizing properties of both antioxidant and antiseptic substances for the activity of thromboplastin suspensions prepared from horse and rabbit brain.

#### EXPERIMENTAL RESULTS

The influence of various substances on the stability of thromboplastin suspensions of horse and rabbit brains were studied at room temperature ( $28^{\circ}\text{C.} \pm 4^{\circ}\text{C.}$ ) in order to simulate the conditions under which these reagents are used in the laboratory. The substance under investigation for its stabilizing action was dissolved in distilled water and added to the thromboplastin suspension (equal volumes). A control suspension diluted with an equal volume of water but containing no stabilizing agent was included in each experiment. The control and treated suspensions were placed in stoppered vials or test tubes, and their thromboplastic activity obtained at intervals by determining the clotting time of citrated human plasma by the one-stage method of Quick. Since all the factors involved in this determination except the thromboplastin suspension were constant, the clotting time of the plasma was taken as a measure of thromboplastic activity.

*Stabilization of Thromboplastic Activity with Hydroquinone.* The addition of 0.5% hydroquinone to a suspension of horse brain thromboplastin produced a marked increase in its stability. Whereas the control suspension lost considerable activity in a few days, the clotting time of the treated suspension remained at its initial level for 5 days in spite of the fact that, at the start of the experiment, the hydroquinone caused the clotting time to increase by 2 seconds over that

of the control suspension. This slight initial inhibition produced by the hydroquinone was not obtained in all such preparations.

The optimal stabilizing concentration of hydroquinone likewise was determined with horse brain thromboplastin (Fig. 1). In evaluating these optimal concentrations two factors were considered, namely, the influence of the agent on the initial clotting time, and the length of time during which the clotting time was maintained at the initial control level. Very low concentrations of the antioxidant (between

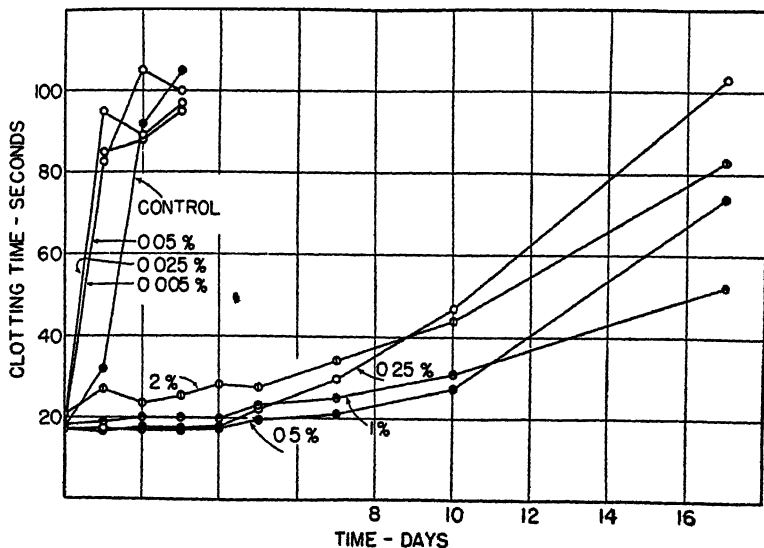


Fig. 1

The Effect of Various Concentrations of Hydroquinone on the Activity of Thromboplastin Suspensions of Horse Brain Powder Stored at Room Temperature

0.005% and 0.05%) were ineffective. In fact, these concentrations appeared to accelerate the destruction of thromboplastic activity. Suspensions containing 0.25% to 2.0% all exhibited a marked stability in comparison to the control. In this experiment a slight inhibitory effect on the clotting time was produced by the presence of 1.0% and 2.0% hydroquinone. No such inhibition was observed with the optimal concentration of 0.25% and 0.5% hydroquinone, which stabilized the thromboplastic activity at the initial level of the control for a period of 4 days.

The protective action of hydroquinone was not displayed in a thromboplastin suspension of horse brain held at 56°C. in a water bath (Table I). At this temperature, control and treated suspensions both lost their activity quite rapidly; in fact, the destruction of thromboplastic activity was accelerated slightly by the presence of the hydroquinone. Apparently the mechanism involved in the destruction of thromboplastin at 56°C. is different from that of storage at room temperature.

TABLE I

*Inactivation of Horse Brain Thromboplastin Suspension at 56°C.*

Time—Minutes	Clotting Times (seconds)	
	Control	0.5% Hydroquinone
0	16.3	16.6
15	18.3	20.1
30	20.1	22.3
60	24.3	27.2
120	40.7	47.2

*Stabilization of Activity with Other Antioxidants and with Antiseptics.* Early in these studies the observation was made that suspensions of thromboplastin which had lost most of their activity after standing at room temperature had putrified. The possibility that the destruction of thromboplastin was caused by the growth of microorganisms seemed plausible, because aseptic technics were not employed in the preparation of either the dry brain powders or their suspensions, and bacteriological tests showed the presence of both gram-positive and gram-negative bacilli and yeasts in the dry brain powders. Therefore, the effects of antiseptics on the stability of thromboplastin suspensions were investigated.

Tables II and III list the various agents used in these studies with their concentrations, and summarize the results of 5 experiments. The substances tested are arranged in the order of their effectiveness, which arbitrarily was measured by the number of days required for the clotting time of each treated suspension to increase 4 seconds over the clotting time of the same suspension before storage at room temperature. The variation in the clotting time produced by the

TABLE II

*The Stabilizing Effect of Various Agents on the Activity of Horse Brain Thromboplastin Suspensions*

Agent	Experiment No.	Concentration per cent	Clotting Time (seconds) of suspension at zero time		Stability Index* Days
			Control	Treated	
1. Phenylmercuric nitrate	5	0.01	17.3	17.4	31
2. Sodium ethylmercurithiosalicylate	4	0.01	17.4	17.8	16
3. Hydroquinone	5	0.5	17.3	18.2	16
	1		17.4	19.2	10
	2		17.4	16.8	7
	3		16.7	17.3	6.5
4. Mercuric chloride	5	0.1	17.3	25.9	14.5
5. Catechol	4	0.5	17.4	18.6	11
6. Pyrogallol	4	0.5	17.4	18.0	10
7. Nordihydroguaiaretic acid	5	0.1	17.3	16.9	9
8. Sulfanilamide + Hydroquinone	3	0.8+0.5	16.7	19.5	9
9. Sulfanilamide	3	0.8	16.7	17.8	8
10. Phenol + Hydroquinone	3	0.25+0.5	16.7	18.5	5.5
11. Phenol	3	0.25	16.7	16.8	5
12. Hexylresorcinol	5	0.1	17.3	22.6	2.5
13. Tyrothrycin	5	0.01	17.3	17.4	2
14. Sodium Propionate	4	0.8	17.4	17.7	2
15. Thymol	5	sat.	17.3	19.6	1.5
16. $\alpha$ -Tocopherol	5	sat.	17.3	17.5	1
17. Controls (no agent added)	1	—	17.4	—	1
	2	—	17.4	—	0.5
	3	—	16.7	—	0.5
	4	—	17.4	—	0.5
	5	—	17.3	—	2.5

\* Days required for clotting time to increase by 4 seconds over the initial clotting time of the treated suspension.

presence of some of these agents was eliminated by determining the index of stability in this manner. Except for hydroquinone, the optimal concentration of the stabilizing substance may not have been employed. However, an attempt was made to use those concentrations commonly employed for preventing contamination (1, 2, 4, 11, 12 in Table II, and 3, 4 in Table III), or, as in the case of nordihydroguaiaretic acid, were considered optimal for their antioxidant effects.

The limiting solubility of other substances (9, 13, 14, 15, 16 in Table II and 5 in Table III) determined the concentration employed. The stabilizing effect of nordihydroguaiaretic acid and phenylmercuric nitrate, illustrated in Fig. 2, exemplify the results obtained in a typical experiment.

TABLE III

*The Stabilizing Effects of Various Agents on the Activity of Rabbit Brain Thromboplastin Suspensions*

(Experiment No. 5)

Agent	Concentration per cent	Clotting time (seconds) of treated suspen- sions at zero time	Stability Index* Days
1. Hydroquinone	0.5	12.3	28
2. Nordihydroguaiaretic acid	0.1	12.3	21
3. Hexylresorcinol	0.1	14.7	20
4. Phenylmercuric nitrate	0.01	12.8	16
5. Thymol	Sat.	11.8	5
6. Control	—	12.3	1

\* Days required for clotting time to increase by 4 seconds over the initial clotting time of the treated suspension.

The results of these experiments indicated that certain antioxidants and antiseptics were potent stabilizing agents. For horse brain thromboplastin suspensions, the mercurial antiseptics appeared to be somewhat more effective than the antioxidants, but the reverse seemed to be true for rabbit brain thromboplastin suspensions. In view of the variability of the results obtained with hydroquinone (experiment 1, 2, 3, 5 in Table I), no claims can be made for the exact order of stabilizing power. However, the most effective antiseptics and antioxidants appeared to be phenylmercuric nitrate, sodium ethylmercurithio-salicylate, hydroquinone, nordihydroguaiaretic acid, hexylresorcinol and mercuric chloride. The two latter substances are effective, but are not desirable stabilizers as they exert an appreciable inhibitory action on the blood clotting mechanism. The inability of thymol and  $\alpha$ -tocopherol to stabilize thromboplastin suspensions may have been due to their low solubility in water.

## DISCUSSION

The experiments on the protective action of antioxidants and antiseptics did not identify clearly the mechanism responsible for the destruction of thromboplastic activity observed in suspensions held at room temperature. Since antioxidants and antiseptics were effective, it would appear that two factors were involved, namely, bacterial

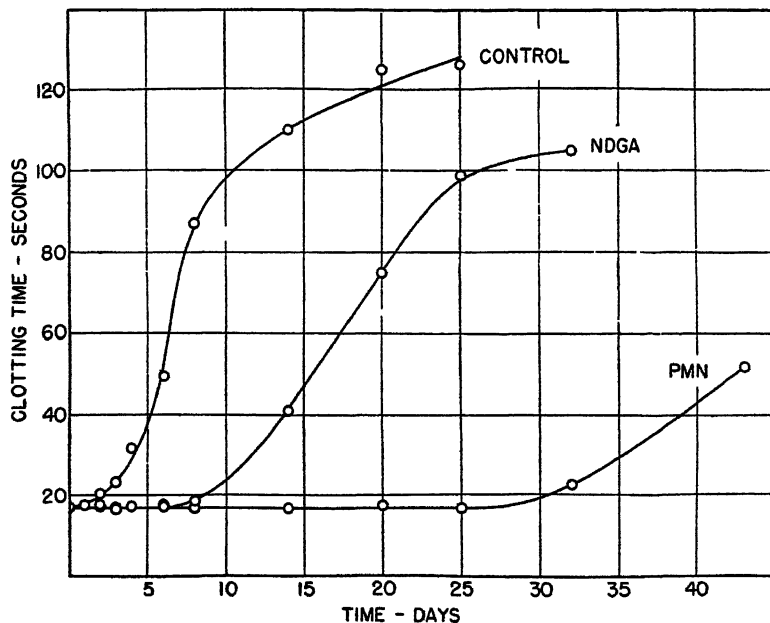


FIG. 2

The Stabilizing Effect of 0.1% Nordihydroguaiaretic Acid (NDGA) and 0.01% Phenylmercuric Nitrate (PMN) on the Thromboplastic Activity of Saline Suspensions of Horse Brain Stored at Room Temperature

destruction and oxidation. Apparently, the microbiological factor has not been recognized before. On the other hand, the oxygen-lability of purified solutions of lung thrombokinase was observed by Marx and Dyckerhoff (8), who were able to stabilize their solutions for a period of 10 months by adding 1% sodium thiosulfate and storing the treated solution in sealed ampoules. In our experiments, the weight of evidence favors the hypothesis that bacterial destruction is the important



factor. Two lines of evidence may be cited: (1) phenylmercuric nitrate, which is not an antioxidant, is an excellent stabilizing agent, and (2) most antioxidants of the quinone type exert an antiseptic action as well as an antioxidant action (9). Therefore, the results could reasonably be explained by assuming that the antioxidants prevented the growth of the microorganisms responsible for the destruction of the active clotting substance. This point of view is well supported by the observation that large numbers of organisms appeared in most suspensions about the time that activity started to decline. Gram-stained slides prepared from these suspensions revealed the presence of gram-positive and gram-negative bacilli and cocci, yeasts and molds, and, in many instances, combinations of these types. Simultaneously with the appearance of large numbers of these organisms the suspensions became putrid and reacted alkaline. Exceptions to these observations were found in those suspensions treated with thymol, hexylresorcinol and mercuric chloride. The loss of thromboplastic activity in these was presumed to be the result of a chemical action.

The possible role of oxidation as a supporting mechanism for the inactivation of thromboplastin cannot be entirely disregarded, because under identical conditions antioxidants appeared to be somewhat more effective stabilizing agents for rabbit brain thromboplastin than for horse brain thromboplastin, although this may have been due to the presence of a bacterial contaminant(s) of greater or lesser resistance to a specific stabilizing agent. A few attempts were made to study the stabilizing effect of antioxidants in sterile suspensions of thromboplastin, *i.e.*, under conditions in which the microbiological factor would be eliminated. Unfortunately, the sterilization of a thromboplastin suspension (horse brain) was always accompanied by some loss of activity. Filtration through Seitz and Hormann sterilizing pads resulted in complete loss of activity. Steam sterilization produced only partial destruction; such sterile suspensions coagulated human plasma in approximately 30 seconds. It was necessary to employ very short periods of exposure to the steam (0 to 15 lbs. pressure; total exposure time, 5 minutes); otherwise the loss of activity was too great. These sterile, but partially inactivated, suspensions did not prove to be at all stable at room temperature. The presence of 0.1% nordihydroguaiaretic acid in such sterile suspensions appeared to offer some protection (about 3 days). It is doubtful whether any conclusions can be drawn from these experiments with sterile suspensions as to the

role of microbiological destruction or non-biological oxidation in the inactivation of thromboplastic suspensions, since the influence of high temperature on the properties of aged thromboplastic suspensions are not known.

Two of the better stabilizers, nordihydroguaiaretic acid and sodium ethylmercurithiosalicylate, were studied to determine whether their presence would interfere with the determination of prothrombin by the one-stage method of Quick. The clotting times of various dilutions of citrated human plasma representing concentrations between 100% and 5% prothrombin were essentially the same at each prothrombin level, regardless of the presence or absence of these substances. The average difference in the clotting times between the control and stabilized thromboplastin preparations for all levels of prothrombin was 5.4% (range - 7.0 to + 7.0%). This indicated that these two substances probably will not interfere with the clinical determination of prothrombin.

#### EXPERIMENTAL DETAILS

The acetone-dried rabbit brain powder was prepared according to the method of Quick (10); the acetone-dried horse brain powder, by our modification of Quick's procedure (11). Saline suspensions of each thromboplastin were prepared as described by Quick (1), except that the suspensions were centrifuged for 30 seconds. The substances to be studied for their stabilizing capacity were dissolved in distilled water (except for  $\alpha$ -tocopherol and thymol) and added to the suspension of thromboplastin (equal volume), so that the concentration of thromboplastin in the final suspension was half that used by Quick. Control suspensions were diluted with an equal volume of distilled water. The clotting times of the rabbit brain suspensions did not change upon dilution with water but, as a rule, horse brain thromboplastin suspensions are more active after dilution with an equal volume of distilled water (12).

Clotting time determinations were made by the one-stage method of Quick (1) using a 0.184% calcium chloride solution, the optimal concentration for our plasma (12). One sample of pooled (10 bleedings) citrated human plasma (50 cc. 4.0% sodium citrate + 500 cc. of blood), preserved in the frozen state at - 20°C., was employed in all experiments. The prothrombin time of the frozen plasma remained constant in the range of 16.5 to 17.5 seconds when horse brain thromboplastin was used.

#### SUMMARY

1. The stability of thromboplastin suspensions of horse and rabbit brains stored at room temperature was investigated. The majority of these suspensions lost considerable activity in 1 day or less.

2. Antioxidants and antiseptics stabilized the suspensions for various periods of time. The most effective agents were phenylmercuric nitrate, sodium ethylmercurithiosalicylate, hydroquinone, nordihydroguaiaretic acid, hexylresorcinol and mercuric chloride. The last two substances were not considered satisfactory stabilizing agents, because they produced a large initial loss of activity, making such suspensions of little value as thromboplastic reagents for the determination of prothrombin.

3. The results indicated that the inactivation of thromboplastin was due to its destruction by microorganisms, since antiseptics and antioxidants possessing antiseptic properties were effective stabilizing agents. The demonstration of the presence of microorganisms in most of the suspensions and the correlation of loss of activity with the appearance of large numbers of organisms strongly support this view. The possible role of oxidation as an additional factor involved in the loss of thromboplastic activity was discussed.

4. Hydroquinone does not prevent the rapid loss of thromboplastic activity taking place in suspensions heated at 56°C.

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## Horse Brain Thromboplastin

### IV. Stabilization of Activity of Dried Brain Preparations\*

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#### INTRODUCTION

The thromboplastic activity of dried brain preparations, from which active blood clotting extracts can be prepared for the determination of prothrombin, gradually declines at room temperature in the presence of air. According to Quick the activity of acetone-dried rabbit brain can be maintained indefinitely by storing the brain powder in the absence of air either under nitrogen or *in vacuo* (1, 2a). Apparently, better results were obtained by employing refrigerator temperature as well as vacuum (2b). The loss of potency in the presence of air was ascribed to the oxidation of an unsaturated component of the cephalin moiety of the thromboplastic molecule. Thromboplastic saline extracts desiccated by the lyophile technic and maintained under vacuum (3, 4) and at 5°C. under vacuum (5) likewise were stable. Desiccated rabbit brain has been stored in tightly-stoppered bottles at refrigerator temperatures without loss of potency for several months (6, 7), while similar preparations lost activity after 154 days of storage at room temperature (6).

The widespread use of thromboplastin in clinical and research laboratories prompted us to study means of stabilizing the activity of dry preparations. From the practical viewpoint, the conditions of

\* Some of the data in this paper were presented at the meetings of the Philadelphia Section of the American Chemical Society on July 13, 1945.

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storage described above, *i.e.*, vacuum, nitrogen or low temperatures, apparently are satisfactory; at least no objections have been noted. That these conditions could be improved might be surmised from the recommendation of Quick (2, b) that both vacuum and low temperature should be used for storage. In view of the instability of thromboplastin on storage in the dry state, it was felt that a search for suitable stabilizing agents might prove of interest.

### EXPERIMENTAL RESULTS

The investigation of the stability of horse brain thromboplastin followed two lines of approach, namely, (1) preservation of thromboplastic activity by exclusion of air from the storage container, and (2) preservation of activity in the presence of the limited quantity of air present in the container by means of antioxidants. The thromboplastic activity of the stored samples of dried horse brain powder was determined at intervals by obtaining the clotting time of citrated human plasma by a modification of the one-stage method of Quick (9). Since the thromboplastin was the only variable in the test, the clotting times obtained were taken as a measure of thromboplastic activity.

*Stability under Vacuum and Gas.* The activity of horse brain thromboplastin stored as a dry powder under vacuum, carbon dioxide and

TABLE I

*The Stability of Horse Brain Thromboplastin Powder Stored at Room Temperature and 5°C. Under Vacuum and Certain Gases in Sealed Glass Tubes*

Storage Time in Days	Clotting Time (Seconds)									
	Vacuum 5°C. R.T.		Nitrogen <sup>1</sup> 5°C. R.T.		Nitrogen <sup>2</sup> 5°C. R.T.		Nitrogen <sup>3</sup> 5°C. R.T.		Carbon Dioxide 5°C. R.T.	
0	16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.8
30	17.0	16.7	17.4	16.9	17.4	17.5	16.7	16.6	16.3	16.5
60	16.9	16.0	16.2	19.6	16.7	18.6	16.3	16.8	17.4	16.9
127	16.3	16.3	17.0	23.8	16.8	21.1	16.6	17.7	16.4	17.3
181	16.2	16.0	16.5	22.0	16.0	22.2	15.9	17.2	16.5	17.5
309	17.1	—	18.4	24.8	18.6	23.3	17.1	18.8	16.8	22.3
414	18.5	—	16.5	27.7	16.3	25.8	15.2	23.3	15.9	17.6

<sup>1</sup> Each tube was alternately evacuated and filled with nitrogen three times before sealing.

<sup>2</sup> Each tube was evacuated and filled with nitrogen once before sealing.

<sup>3</sup> The air in each tube was merely displaced with nitrogen.

nitrogen in sealed glass test tubes at 5°C. and room temperature was studied over a period of approximately one year (Table I). At 5°C. the thromboplastin proved to be very stable; there was essentially no change of activity in any group of samples during this time. However, at room temperature some loss of activity was found in those samples stored under nitrogen. Peculiarly enough, in the groups of samples where an attempt had been made to remove the last traces of air by alternately evacuating and filling the container with nitrogen a number of times, the thromboplastin proved to be less stable than in the group where the air was merely displaced with nitrogen at atmospheric pressure. At room temperature in the presence of carbon dioxide or vacuum the brain powder retained its activity at approximately its initial level.

*Stabilization with Antioxidants.* In the presence of air at room temperature, horse brain thromboplastin eventually lost its activity and, in the course of only a few weeks, could no longer be considered an active clotting agent. This loss of activity was retarded remarkably by the addition of an antioxidant to the dried horse brain powder. The effectiveness of the antioxidant was dependent upon the manner in which it was incorporated in the brain powder. A mixture of hydroquinone and brain powder prepared by thorough grinding in a glass

TABLE II

*The Effect of Hydroquinone on the Thromboplastic Activity of Horse Brain Powder Stored at Room Temperature*

Preparation	Storage Time Days	Clotting Time (Seconds)	
		Control	14.2% Hydroquinone
Hydroquinone mixed with brain powder in a mortar	0	16.3	18.0
	16	19.7	19.7
	32	23.6	20.1
	53	24.7	21.6
	281	48.1	25.4
Hydroquinone added to the brain powder by acetone-vacuum distillation procedure	0	16.2	19.0
	8	19.8	19.1
	24	21.2	19.3
	45	25.0	19.3
	273	45.4	20.5

mortar was more stable than the brain powder itself when both were stored at room temperature in tightly stoppered jars (Table II). However, the stabilizing effect was only temporary. When the dried brain powder was treated with an acetone solution of hydroquinone, and the acetone subsequently removed by vacuum distillation, the resultant mixture, in which the hydroquinone was dispersed homogeneously throughout the brain powder, retained its thromboplastic

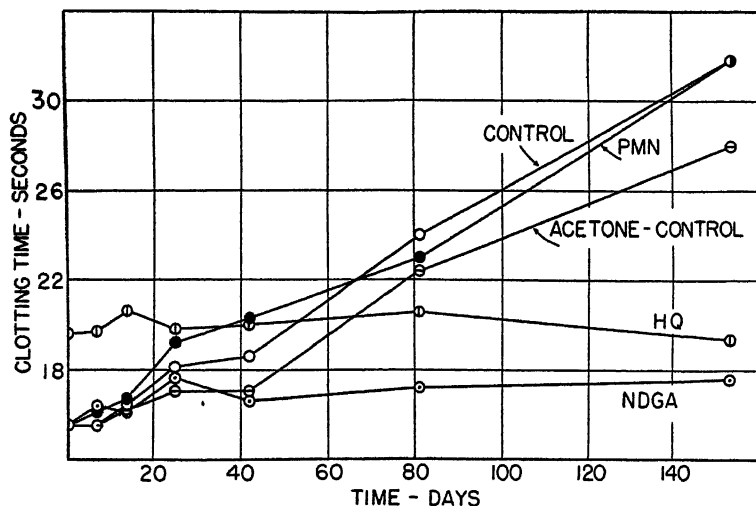


FIG. 1

The Effect of 14.2% Hydroquinone (HQ), 3.2% Nordihydroguaiaretic acid (NDGA) and 0.33% Phenylmercuric Nitrate (PMN) on the Thromboplastic Activity of Acetone-Dried Horse Brain Powder Stored at Room Temperature. The curves designated as control and acetone control represent, respectively, untreated acetone-dried horse brain and acetone-dried horse brain subjected to the same acetone treatment used to incorporate the stabilizing agents.

activity for at least 9 months. The clotting times obtained with the latter preparation were a few seconds higher than that of the control at zero time but, nevertheless, remained at this level unchanged for 9 months.

Other experiments, in which the acetone procedure for adding antioxidants to dry brain powder was employed, substantiated the stabilizing effect of hydroquinone and certain other antioxidants. The course of a typical experiment is illustrated in Fig. 1. Nordihydro-

guiaretic acid (NDGA), an excellent antioxidant for fats and oils (10), was as effective as hydroquinone and, in addition, did not produce the slight initial increase in the clotting time that was almost always observed with hydroquinone. Control preparations of dried horse brains, including one that had been subjected to the acetone treatment in the absence of any antioxidant (acetone-control), rapidly lost their activity in comparison to the antioxidant-containing powders. Phenylmercuric nitrate (PMN), a substance that presumably has no antioxidant powers, was included in this experiment in order to contrast its role in the stabilization of saline suspensions of thromboplastin (11) with its expected lack of stabilizing power in dry thromboplastin preparations, and to serve as an additional control for the experiment. It did not prove to be a stabilizing agent for the dried brain powder.

In general, the effects observed above were confirmed in other experiments (Table III). NDGA and hydroquinone afforded complete protection for as long as 9.5 months. A reduction in the hydroquinone concentration from 14.2% to 7.6% did not impair its ability to preserve activity for at least a period of 5.5 months, and furthermore did not cause the clotting time to increase as did the higher concentration. However, *l*-ascorbyl palmitate, a stabilizer for fats and oils (10), did not appear to be an effective agent for horse brain thromboplastin. In fact, it accelerated the destruction of thromboplastic activity and, in combination with NDGA, it partly nullified the effectiveness of the latter. The antiseptic substance, sodium ethylmercurithiosalicylate (SET), appeared to act in somewhat the same manner as phenylmercuric nitrate, described above. The presence of phenylmercuric nitrate and sodium ethylmercurithiosalicylate in powders containing NDGA did not impair or improve the stabilizing action of the latter substance.

The optimal concentration of the stabilizing agents studied here was not determined. The concentration employed in the dry powder was in some cases based on the experimental data obtained in the preceding paper (11), the amount of agent incorporated in the dry powder being such that the final concentration in the saline suspension was approximately the same as that previously used for the saline suspensions. However, the antiseptic substances in most instances were added to the brain powder in such quantities that their concentration in the suspension was approximately ten times that previously employed.



TABLE III

*Stabilization of Thromboplastic Activity of Desiccated Horse Brain*

Experiment Number	Stabilizing Agent*	Per cent agent in dry brain powder	Clotting Time (Seconds)		Duration of Experiment in Months
			At Start	At Conclusion	
1	None	—	15.5	86.3	9.5
	HQ	14.2	19.6	17.4	
	NDGA	3.2	15.6	16.9	
	PMN	0.3	15.6	79.0	
2	Control	—	16.7	40.8	5.5
	HQ	7.6	16.9	17.2	
	NDGA	3.2	16.7	17.2	
	PMN	3.2	16.8	52.9	
	SET	3.2	17.3	43.6	
	NDGA+l-AP	3.2+3.2	18.1	29.7	
	NDGA+l-AP +PMN	$\left\{ \begin{array}{l} 3.2+3.2 \\ +0.3 \end{array} \right\}$	18.1	35.3	
3	Control	—	16.9	46.8	5.8
	NDGA	3.2	16.2	18.1	
	NDGA+PMN	3.2+0.3	15.4	18.0	
	l-AP	3.2	17.1	78.0	
4	Control	—	15.8	35.2	3.0
	NDGA	3.2	15.9	16.3	
	SET	3.2	15.1	27.2	
	NDGA+SET	3.1+3.1	15.4	16.1	

\* The following abbreviations were employed: NDGA = nordihydroguaiaretic acid, PMN = phenylmercuric nitrate, HQ = hydroquinone, l-AP = l-ascorbyl palmitate, SET = sodium ethylmercurithiosalicylate.

It had been demonstrated previously (11) that most of these substances could stabilize thromboplastin suspensions when they were added directly to the final saline suspension of the acetone-dried horse brain powder. The question arose whether these same stabilizing agents, when incorporated in the dry brain powder, would likewise be able to preserve the activity of saline suspensions prepared from the treated brain powders. Accordingly, saline suspensions were made from the preparations listed in Table III, and their stability was determined. *These studies were made on each preparation before it was*

2 days old and not with aged brain powders. Surprisingly enough it was found that suspensions prepared from PMN-, NDGA- and PMN + NDGA-treated brain powders were not very stable, while those prepared from SET-, HQ- and SET + HQ-treated powders had retained their activity as previously observed. *l*-Ascorbyl palmitate-treated brain powders were inactive. Data on NDGA, SET and a mixture of these 2 substances are presented in Table IV. Here the

TABLE IV

*The Stability of Thromboplastin Suspensions Prepared From Horse Brain Powder Treated with NDGA and Sodium Ethylmercurithiosalicylate (SET)*

Time in Days	Clotting Time (Seconds)			
	Acetone Control	NDGA	SET	NDGA + SET
0	15.8	15.9	15.1	15.4
5	16.3	16.1	16.8	16.4
8	73.6	36.5	16.5	16.7
14	90.7	85.3	16.5	16.1
19	89.0	79.0	16.0	18.4

loss of activity in the case of NDGA is the same as that of the control, while the presence of SET provided a stabilized suspension either in the presence or absence of NDGA for at least 14 days. Incidentally, the control was active for 5 days, which was the longest fully active period ever observed by us for an untreated horse brain thromboplastin suspension. Most suspensions do not last longer than 1 to 2 days.

## DISCUSSION

Horse brain thromboplastin, like rabbit brain thromboplastin, can be stored under vacuum either at refrigerator or room temperatures without loss of activity. Our experiments indicate that carbon dioxide is equally effective. Nitrogen was not very satisfactory for preserving the activity of desiccated horse brain stored at room temperature, but this may have been due to the oxygen content of commercial nitrogen. At refrigerator temperatures similar preparations stored under nitrogen were stable. In our experience the destruction of thromboplastin in the presence of the oxygen of air is practically zero at low temperatures. For example, acetone-dried preparations of

horse and rabbit brain have been stored at  $-20^{\circ}\text{C}$ . for periods of two and five years respectively with no loss of thromboplastic potency, whereas at room temperature destruction was comparatively rapid. The importance of low temperatures for the preservation of thromboplastin is amply borne out by these observations and those of others (6, 7).

The loss of thromboplastic activity that takes place at room temperature can be prevented for at least 9.5 months by incorporating an antioxidant such as hydroquinone or NDGA in the dry brain powder. A homogeneous distribution of the antioxidant was essential for effective stabilization. For practical purposes, either 3.2% NDGA or 7.6% hydroquinone appeared to be satisfactory. The color of these stabilized powders did not change, whereas preparations containing inactive stabilizing agents, such as *l*-ascorbyl palmitate, phenylmercuric nitrate, and sodium ethylmercurithiosalicylate gradually turned to a darker tan or brown color.

The increased destruction of thromboplastic activity produced by *l*-ascorbyl palmitate remains unexplained. Saline suspensions of brain powder containing this substance were slightly more acid (pH 5.4) than control suspensions (pH 6.5). This suggested that perhaps a splitting of the antioxidant molecule into palmitic and ascorbic acid took place in stored suspensions. It has been demonstrated that an optimal pH level for the determination of the clotting time by the one stage method of Quick (12) exists. The possibility that the low pH of the suspension from the aged *l*-ascorbyl palmitate preparation (6.5 months old) was responsible for the abnormally prolonged clotting time was checked. Adjustment of the pH of the suspension from 5.36 to 6.8 changed the clotting time from 89.4 seconds to 79.6 seconds. Apparently the acid reaction observed with this preparation did not greatly influence the results obtained in the stability studies. The pH change found in the *l*-ascorbyl palmitate preparations was not observed in other preparations except in the control and in sodium ethylmercurithiosalicylate-treated preparations of experiment 2 (Table III), where the pH dropped as low as 6.0. Adjustment of the pH to a normal value (6.5) did not influence the clotting times of these preparations.

The stabilization of dry thromboplastin powders offers the investigator and clinician a preparation with constant activity, or at least one that is much less apt to change its activity on storage. This is

an advantage in so far as it tends to eliminate the necessity for too frequent checks on the activity of a particular batch of thromboplastin. An additional advantage may be found in the thromboplastins containing both antioxidants and antiseptics. Our experiments indicated that, by incorporating a suitable antiseptic with the antioxidant, the saline suspensions ultimately prepared from these preparations were stabilized for two weeks. Sodium ethylmercurithiosalicylate proved to be a suitable antiseptic for this purpose while phenylmercuric nitrate did not. The lack of stabilizing power for saline suspensions exhibited by phenylmercuric nitrate and NDGA when incorporated in the dried brain preparations may be the result of poor solubility in aqueous systems, or perhaps may be due to their adsorption on some of the protein that is removed from the suspension by centrifugation.

The stabilization of the acetone-dried brain powder by means of antioxidants indicates that oxidation is involved in the loss of thromboplastic activity on exposure to air at room temperature. Lein and Hays (8) recently reported that hydroquinone preserved the thromboplastic activity of phospholipid preparations of beef brain which were subject to loss of thromboplastic activity on standing under similar conditions.

### EXPERIMENTAL DETAILS

The acetone-dried horse brain powder was prepared according to our modification of Quick's procedure for rabbit brain (9).

In the experiments involving the preservation of thromboplastic activity under vacuum and gases, 120 mg. quantities of desiccated brain powder were stored in flame-sealed pyrex glass test tubes. Vacuum of a few minutes duration was supplied by a 'Cenco' Hyvac Pump. Nitrogen and carbon dioxide were introduced into the tubes by alternately producing a vacuum and allowing the gas to flow in, followed by evacuation and refilling for a total of three such cycles. In one case, only two cycles were employed for nitrogen and, in another, the air was merely displaced from the tube with nitrogen at atmospheric pressure. The sealed tubes were stored at 5°C. and room temperature, and their activity determined at intervals.

For studies of thromboplastic activity at room temperature in the presence of air, 10 g. aliquot portions of acetone-dried horse brain powder were suspended in 25 to 50 cc. of acetone solutions of the stabilizing substances studied. The mixture was thoroughly agitated for 10-30 minutes in a stoppered round bottom flask and the acetone removed by vacuum distillation. As additional assurance that all of the acetone was removed, the preparations were held in a vacuum oven overnight at room temperature. The dried preparations were stored in tightly stoppered jars closed with metal caps containing waxed linings. The stabilizing agents studied

were soluble in acetone, except phenylmercuric nitrate, which was only partially soluble. In the latter case, the PMN suspension in acetone was warmed a little to increase slightly the solubility of the antiseptic substance.

Thromboplastic activity was determined by the one-stage method of Quick (13), except that in the preparation of the suspension a shorter period of centrifugation (30 seconds) was employed, and the resultant extract was diluted with an equal volume of water. 0.184%  $\text{CaCl}_2$  solution was used instead of 0.277% as recommended by Quick (5). The dilution is necessary for the preparation of the most active saline suspensions when horse brain thromboplastin is used (5). Six *per cent* suspensions of stabilized brain powder were employed in all experiments, except those listed under experiments 2 and 3 in Table III, where corrections were made for the presence of the stabilizing agents. This appeared to be unnecessary, however. Clotting time determinations throughout were made with one pooled sample (10 bleedings) of citrated human plasma (50 cc. 4.0% sodium citrate + 500 cc. blood) preserved in the frozen state at  $-20^\circ\text{C}$ . in 10 cc. aliquot portions. The clotting time of this plasma remained constant throughout the entire period of investigation.

In experiments on the stability of saline suspensions prepared from the stabilized thromboplastin preparations, the diluted saline suspensions were stored in stoppered test tubes at room temperature, and their activity determined at intervals.

### SUMMARY

1. The activity of desiccated horse brain thromboplastin has been preserved by storage under vacuum or carbon dioxide at  $5^\circ\text{C}$ . and at room temperature. Nitrogen was satisfactory at refrigerator temperatures but not at room temperature.

2. At room temperature, in the presence of air, the loss of thromboplastic activity was comparatively rapid. The addition of certain antioxidants to the desiccated thromboplastin preserved its activity for at least 9.5 months. Nordihydroguaiaretic acid (3.2%) and hydroquinone (7.6%) proved to be excellent stabilizing agents. *L*-Ascorbyl palmitate and, as might be expected, phenylmercuric nitrate and sodium ethylmercurithiosalicylate were without activity.

3. The addition of sodium ethylmercurithiosalicylate to the NDGA stabilized brain powders enhanced their usefulness by stabilizing the saline suspensions prepared from the dry powders. Phenylmercuric nitrate and NDGA, both active stabilizers when added to thromboplastic suspensions, were essentially without activity in suspensions prepared from dried brain powder to which these substances had been added.

4. The studies with antioxidants support the view that oxidation is involved in the loss of thromboplastic activity in the presence of air.

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# Sodium-Retaining Substances of the Adrenal

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## INTRODUCTION

The adrenal cortex is so essential for the maintenance of a normal plasma sodium level that, without it, the loss of sodium may lead to serious consequences in most mammals. Extract which will replace this function is readily prepared from the adrenal. However, none of the crystalline compounds which have been isolated from the gland will account for its sodium-retaining power. Corticosterone and dehydrocorticosterone are not sufficiently potent in this respect, while desoxycorticosterone, if it occurs at all, is present in amounts entirely too small. Moreover, desoxycorticosterone (DC) has been shown to produce metabolic disturbances and pathological changes which have not been obtained from adrenal extract. Evidence has already been offered that the sodium-retaining substance, which exists normally in the unfractionated extract, is distinct from other physiologically active substances (1, 2).

In the present study, methods designed to produce the least amount of chemical change have been used to separate the sodium-retaining substances from the glyconeogenetic substances. The most potent sodium-retaining substance obtained exceeded the potency of DC and differed from it in both chemical and pharmacological properties. This substance is rather readily destroyed by chemical treatment such as has been employed by some investigators in the fractionation of adrenal extracts, which may thus explain their failure to obtain it.

## METHODS

Two methods of assay have been employed in following the fractionation of the biologically active substances obtained from the adrenal:



one, the ability to cause retention of sodium, and the other, the ability to cause glycogen deposition in the liver of a starved animal.

*Sodium retention.* This was determined by the method of Hartman, Lewis and Thatcher (3), except that drinking water was removed from the cage one hour after feeding. This gave more constant assays. Control tests were run daily until the control level was essentially constant.

*Glycogen deposition.* The method employed for this assay is similar to that described by Olson *et al.* (4), but we used fasted normal rats on a low protein diet. Our unit of activity is the amount of material which will cause an increase in liver glycogen of 5 mg./g. of liver over the control level.

#### *Preparation of Concentrates*

A modification of Cartland and Kuizenga's (5) method has been used in the preparation of concentrated extract from the whole adrenal gland. Ethyl alcohol instead of acetone was used for the initial extraction since the yield of sodium-retaining substances was found to be higher by this method.

Frozen beef glands were ground into chilled 95% ethyl alcohol (1.5 liters per kg. of tissue) and shaken under vacuum for eight hours in a stoneware churn. The mixture then stood for 12 hours at 4°C., after which the extract was separated in a press and then reduced to small volume *in vacuo* at 35°C. The aqueous residue was extracted with petroleum ether to remove inert fatty material and phospholipids. The active substances were extracted from the aqueous concentrate by means of ethylene dichloride. This left behind epinephrine and inert substances. Water was removed from the ethylene dichloride by freezing and filtering. The ethylene dichloride was removed by vacuum distillation. The residue was taken up in ethyl alcohol and the remaining fat and cholesterol were removed by partitioning between aqueous alcohol and petroleum ether. The final concentrate was stored in 70% alcohol at -20°C. until several lots had accumulated, when they were combined and concentrated to an approximately 10% alcoholic solution made so that each cc. represented 100 g. of tissue. Sodium chloride was added to make a 0.9% solution. Chilling of this solution precipitated a dark tarry material which was filtered off. This residue was redissolved in 95% alcohol and diluted to make a 10% alcohol and 0.9% sodium chloride solution. Chilling again separated a tarry mass. Repetitions of this procedure, three or four times, removed sodium-retaining substances which were caught when the precipitate was formed. The active 10% alcoholic extracts were combined and extracted with one-fourth volume of ethylene dichloride. After removal of the water from the ethylene dichloride by freezing and filtering, the ethylene dichloride was removed by vacuum distillation, leaving a yellow resinous material. This was the starting material for the present investigations.

#### *Purification by Precipitation*

After numerous trials, it was found that, by dissolving the whole concentrate in chloroform and adding nine volumes of ethyl ether, a precipitate was formed which had a greater potency per mg. than the original. Repeated precipitation resulted in further increase in potency. Maximum fractionation was obtained when the concentration of solid was 5-10% of the total solvent mixture. The temperature at

which the precipitation was carried out played a rôle in the degree of purification. Low temperatures resulted in precipitates which contained a large proportion of the original activity but demonstrated a relatively small increase in potency in terms of units/mg. Higher temperatures gave preparations with large increase in potency but only a fraction of the original activity. The increase in potency can be illustrated by the following example: original concentrate, 1 to 2 units/mg.; first ppt. at  $-20^{\circ}\text{C}.$ , 2.7 to 3.6 units/mg.; second ppt. at  $10^{\circ}\text{C}.$ , 6.8 to 11.4 units/mg.; third ppt. at  $10^{\circ}\text{C}.$ , 11.3 to 16.5 units/mg.; fourth ppt. at room temp., 19.5 to 23.6 units/mg. Preparations assaying as high as 27 units/mg. were obtained. (DC—14.3 units/mg.)

As the potency increased with repeated precipitations, the material became less soluble in chloroform, ethylene dichloride, benzol and acetone. For this reason the concentration of solid in the later precipitation steps was reduced to 2% of the total volume of solvent. Solvent precipitation resulted in the scattering of a large portion of the total activity through the various fractions. The most potent precipitate was an amorphous powder which produced no effect on potassium excretion. DC could not be the substance in the precipitate responsible for sodium retention since it is quite soluble in ethyl ether and one would expect to find it in the filtrates rather than in the precipitates.

#### *Molecular Distillation*

The dry concentrate was fractionated by distillation in a vacuum at  $10^{-6}$  mm. Hg. Fractions were removed at temperatures ranging from  $70^{\circ}$ – $200^{\circ}\text{C}.$  These were assayed for sodium-retaining potency. No fractions distilling at a temperature lower than  $120^{\circ}\text{C}.$  showed this potency. Some of the fractions were more potent than DC. One of them assayed 29 units/mg. None of the active fractions produced any effect on potassium excretion. For example: Preparation A, assaying 12 sodium-retaining units/mg., gave 4.62 mEq. of potassium in the urine in a six hour period, while control runs before and after the test gave 3.93 to 4.48; Preparation B, assaying 18 sodium-retaining units/mg., gave 3.62 mEq. of potassium in the urine in a six hour period with controls of 3.02 to 3.95; Preparation C, assaying 28.8 sodium retaining units/mg., gave 1.85 mEq. of potassium in the urine in a six hour period with controls of 1.92 to 2.25. Purification by this method was followed by a decrease in solubility in chloroform, ethylene dichloride, benzol and acetone. Although very potent preparations were obtained by this method, the losses in potent material were too great for continued use. Pure DC distilled between  $90^{\circ}$  and  $95^{\circ}\text{C}.$  at  $10^{-6}$  mm. Hg with no loss in potency.

#### *Chromatographic Adsorption*

A flowing chromatogram was found to be the best method for the separation of the sodium-retaining substances from the other substances in the whole extract. This was conducted in the cold ( $4^{\circ}\text{C}.$ )

using Merck's aluminum oxide as the adsorbent. It was found that either acetone or ethylene dichloride would elute large quantities of inert material together with the carbohydrate active substances and leave over 80% of the total sodium-retaining activity adsorbed upon the column. This sodium-retaining substance was eluted from the column with methyl alcohol. Ethylene dichloride was employed as the initial eluent in the later part of this study in place of acetone (Table I) as the latter formed condensation products which were difficult to remove.

TABLE I  
*Potency of Fractions Separated by Chromatography*

Fraction	Solvent	Solid	Sodium retention units		Glyconeogenetic units	Potency total
			g.	per mg.		
IV-0 (original)		17.4	2.5	43,500	(12) 2.6 $\pm$ 0.9*	45,300 $\pm$ 15,300
IV-1	Acetone	12.65	0.95	12,000	(20) 4.46 $\pm$ 0.89	56,500 $\pm$ 11,300
IV-2	Ethyl alcohol					
	Methyl alcohol**	4.5	10.0	45,200	(8) 0.74 $\pm$ 0.25	3,300 $\pm$ 1,100
IV-3	50% Methyl alcohol	1.75	0.85	1,500	None	None
		18.9		58,700		59,800 $\pm$ 12,400
V-0 (original)		30.5	3.2	97,600	(8) 1.75 $\pm$ 0.59	53,400 $\pm$ 17,900
V-1	Ethylene dichloride	16.17	1.9	30,700	(16) 2.2 $\pm$ 0.35	35,600 $\pm$ 5,700
V-2	Methyl alcohol	9.57	9.8	93,800	(12) 0.71 $\pm$ 0.23	6,800 $\pm$ 2,200
V-3	50% Methyl alcohol	3.32	0.9	3,000	None	None
		29.06		127,500		42,400 $\pm$ 7,900

\* Deviations are reported in terms of the standard error of the mean.

\*\* 1 part EtOH to 2 parts MeOH.

Number of rats shown in parentheses (Col. 6).

The hormone concentrate was adsorbed upon a prepared column of alumina from an ethylene dichloride solution. The column was then continuously eluted with pure ethylene dichloride until no appreciable amount of solid was being removed.

The first fraction (V-1) was a light-yellow oily material, from which, upon concentration to a small volume and standing in the cold, dimethyl sulphone crystallized out. On an average, 190 mg. were obtained from 1000 kg. of adrenal tissue. Piffner and North (6) obtained similar yields of this substance from the adrenal.

The second fraction (V-2) was obtained by elution with absolute methyl alcohol until no solid was being removed. The third and final fraction (V-3) was obtained by eluting the column with 50% aqueous methyl alcohol.

The behavior of desoxycorticosterone (DC) on a column of alumina under the same conditions is quite different from that of the natural sodium-retaining substance. Ethyl ether, acetone and ethylene dichloride, each caused complete removal of DC from the column.

The potency of two concentrates separated by chromatographic adsorption is shown in Table I. IV-0 was the material from 1773 kg. of adrenal tissue; V-0 was from 3636 kg. of adrenal tissue. Fractions IV-2 and V-2 possessed the highest total potency for sodium retention, while IV-1 and V-1 had the highest total potency for glyconeogenesis. The potency/mg. for sodium retention was also greatly increased in fractions IV-2 and V-2, while the potency/mg. for glyconeogenesis was somewhat increased in IV-1 and V-1. The total units of sodium-retaining substances were apparently increased by the fractionation, while total units of glyconeogenetic substances appeared to be increased in the IV series and decreased in the V series. The sodium retention by the dominantly glyconeogenetic fractions IV-1 and V-1 could be accounted for by the presence of the glyconeogenetic substances (corticosterone and dehydrocorticosterone) since it has been shown that these known crystalline compounds also possess the power of sodium retention to some degree. These compounds are known to be present in the whole gland concentrate in sufficient quantities to account for the entire sodium-retaining activity exhibited by these two fractions.

It will be noted in Table I that there is an increase in the total number of sodium-retaining units when the values for the different fractions are added together, as compared with the assay of the original material. This might be explained by the removal of substances which cause increased sodium excretion. Thorn, Engel and Lewis (7) reported that both 17-hydroxycorticosterone and 17-hydroxy-dehydrocorticosterone cause increased excretion of sodium. These

are the most potent glyconeogenetic compounds (8) and hence would be removed with fractions IV-1 and V-1. The discrepancies between the total number of glyconeogenetic units in the original material and the combined fractions may be accounted for by the error of the assay method.

### *Comparison of Relative Solubilities*

The fractions most active in glyconeogenesis were soluble in methyl and ethyl alcohols, propylene glycol, acetone, chloroform, ethylene dichloride, ethyl acetate and benzol; partially soluble in carbon tetrachloride and ethyl ether; insoluble in petroleum ether and water.

The fractions most active in sodium retention were soluble in methyl and ethyl alcohols, propylene glycol, glacial acetic acid and acetic anhydride; partially soluble in acetone; insoluble in chloroform, ethylene dichloride, benzol, carbon tetrachloride, petroleum and ethyl ethers and pyridine. They were slightly soluble in water and insoluble in dilute (0.1 *N*) hydrochloric acid, but were very soluble in dilute (0.1 *N*) sodium hydroxide. DC is soluble in chloroform, ethylene dichloride, benzol, carbon tetrachloride, ethyl ether and pyridine, but insoluble in petroleum ether, just the reverse of the sodium-retaining principle.

If these preparations owed their activity to DC, their solubility would be expected to become more, rather than less, similar to DC upon purification. The solubilities of DC correspond fairly well with those of the glyconeogenetic substances but not so well with those of the sodium-retaining substances of the adrenal (Table II). It should be noted that the solubility of the sodium-retaining substances changes upon purification. There was a decrease in solubility in chloroform, ethylene dichloride, benzol and acetone. This could be explained by the removal of substances which increased their solubility in those solvents.

Exposure of the most potent sodium-retaining fraction to 0.1 *N* HCl for a short time at room temperature changed its character so that it became much more soluble in chloroform, ethylene dichloride and benzol, and almost completely insoluble in water. The solubility in ethyl ether apparently was unchanged. Acid changed the material from a light-yellow amorphous solid to a dark-yellow resin. A large part of the potency was lost. Before acid treatment, assay indicated 10 sodium-retaining units/mg., while after treatment this had fallen to

TABLE II  
*Comparison of Relative Solubilities*

	Ethyl ether	Benzol	Ethylene dichloride	Acetone	Methyl or Ethyl alcohol	Water
Glyconeogenetic substances	++	++++	+++++	+++++	+++++	0
Sodium-retaining substances	0	0	0	++	+++++	++
Desoxycortico-sterone	++++	+++++	+++++	+++++	+++++	0

1.2 units/mg. After exposure to HCl, an appreciable amount of NaCl was isolated from the material.

#### *Acetylation*

The sodium-retaining fraction was easily acetylated. 978 mg. treated with 3.5 cc. of acetic anhydride in 4 cc. of pyridine for 18 hours at room temperature yielded 1210 mg. of semi-crystalline substance after the pyridine and excess acetic anhydride were completely removed under high vacuum (0.003 mm. Hg). A large amount of heat was released in the acetylation. This would seem to indicate active hydroxyl groups. After acetylation, the sodium-retaining units had dropped to 4.5 units (from 10 units)/mg. This was based on the weight of the original material, not on the product of acetylation.

#### DISCUSSION

Chromatographic adsorption seems to be the best method for the separation of adrenal fractions since, in the primary concentrate, it appears to cause little or no loss in either the sodium-retaining or glyconeogenetic activities. It has been used by Reichstein and von Euw (9) and by Mason (10) for the separation of adrenal fractions which had already been exposed to procedures that may have produced considerable loss in potency, since the active substances are sensitive to relatively mild chemical action.

Desoxycorticosterone is the most potent known crystalline compound for sodium retention. 17-Hydroxy-11-desoxycorticosterone also causes retention (11). Corticosterone and dehydrocorticosterone possess one-fourth the activity or less (12). Two other compounds, progesterone and estrone, which have been isolated from the adrenal, have

even less effect on sodium retention (13). None of these would account for the total sodium-retaining potency obtained, since DC, the only compound which approaches our fraction in potency/mg., would have been removed from the column in fractions IV-1 and V-1 by acetone or ethylene dichloride.

The work of Reichstein and von Euw (9) indicates that desoxycorticosterone acetate was removed from a column of alumina before the acetates of those compounds active in carbohydrate metabolism were removed. Strain (14) has pointed out that mixtures of free sterols arrange themselves upon a column in the same order assumed by mixtures of their acetates. Thus, if the whole adrenal concentrate owed its sodium-retaining power to DC, the highly potent sodium-retaining fractions should have been removed from the column before those which were active in carbohydrate metabolism. This was not found to be the case.

It is remarkable that Reichstein and von Euw (9) obtained only 29 mg. of DC/1000 kg. of tissue (our calculations). Perhaps it was not an original constituent but was produced in the process of fractionation.

Our sodium-retaining substance fails to influence potassium, although unfractionated extract has been shown to cause excretion of potassium (17). However, this may be due to corticosterone, dehydrocorticosterone (13), 17-hydroxycorticosterone and 17-hydroxydehydrocorticosterone (7), since they increase potassium excretion. Small amounts of epinephrine may also increase potassium excretion (12).

DC produces pathological disturbances under certain conditions (15), while no such effect of adrenal extract has been demonstrated (16).

The "amorphous fraction" of other workers may have contained the sodium-retaining substance, since it has been described as having the same solubility characteristics (18) as our substance. Moreover, it is said to have no carbohydrate activity (19). However, Kendall (20) found that enormous doses of his amorphous fraction caused no increase in serum sodium concentration.

We are indebted to Parke, Davis and Company, through the courtesy of Doctor Oliver Kamm, for the adrenal glands; and to Doctor R. D. Shaner of Roche-Organon, Inc. for the desoxycorticosterone.

We wish also to thank Doctor Katharine A. Brownell for some of the carbohydrate assays.

## SUMMARY

A substance highly potent in its ability to cause retention of sodium in the body has been prepared in three ways, viz. by fractional solvent precipitation, by molecular distillation and by chromatographic adsorption. The latter appears to produce little loss in potency, which is not true for the other methods. There is great difference in the behavior of this substance and desoxycorticosterone (DC) on a column of alumina. Their solubilities are also quite different. This substance is soluble in methyl and ethyl alcohol, propylene glycol, glacial acetic acid and acetic anhydride, partially soluble in acetone, and insoluble in chloroform, ethylene dichloride, benzol, carbon tetrachloride, petroleum and ethyl ethers and pyridine. It is somewhat soluble in water and insoluble in dilute (0.1 *N*) hydrochloric acid, but very soluble in dilute (0.1 *N*) sodium hydroxide. It distills at a higher temperature than does DC. It has no effect on potassium excretion. It occurs in sufficient amount to account for the sodium-retaining power of adrenal extract.

Treatment with 0.1 *N* HCl destroys a considerable proportion of the potency and forms NaCl. Acetylation destroys part of the potency with evolution of a large amount of heat.

The existence of dimethylsulfone in adrenal extract was confirmed.

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# Physico-Chemical Aspects of the Action of Anion Exchange Resins in Biochemical Systems

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## INTRODUCTION

The discovery by Adams and Holmes in 1935 (1) of ion exchange resins opened the field for the preparation of ion-free water by application of two-step ion exchange. These investigators found that polyhydric phenol-formaldehyde resins, when polymerized to the insoluble C stage, would exhibit the phenomenon known as base exchange. The reaction is governed by the law of mass action and is reversible in accord therewith. Adams and Holmes reasoned that an amino-formaldehyde resin would show removal of acids in consequence of basic groups present in the polymerized resin. In the case of these anion exchangers, the acid adsorbing action is accomplished by amino groups which form a part of the surface of large insoluble organic molecules. In other words, the anion adsorbent is pictured as a high molecular weight compound having throughout the lattice activated amino groups—( $\text{NH}_2$ ). These are capable of forming stable amino hydrochlorides and, thus, the acid is removed molecularly and fixed reversibly.

Matchett (2) has used acid adsorbing resins to isolate tartaric acid. Grape wastes are filtered thru and the acid is adsorbed and subsequently removed with sodium carbonate. Formic acid is removed from formaldehyde in a similar manner (2) resulting in a better product of formaldehyde. Gaddis (3) has used Amberlite IR-4 in an unusual manner. He found that a stable compound was formed with hydrogen sulfide and that the resin would adsorb 12% of its weight of hydrogen sulfide. This addition compound was used in the analysis of Group II ions. Block (4) utilized resinous exchangers in the process of production of arginine, histidine and lysine. Meyers (2) reports that thiamine hydrochloride is adsorbed quantitatively on Amberlite IR-100.

Amberlite IR-4, or as it is now called Amberlite XE-43, is a polyethylene poly-amino methylene substituted resin of diphenyloldimethylmethane and formaldehyde in basic form. De-Acidite, which is also used as an antacid, is an aliphatic amine resin containing principally tertiary amine groups with some primary and secondary groups.

De-Acidite is called an anion exchanger, but the facts show that the free acid molecules react in much the same way that ammonia reacts with an acid (5).

Anion exchange resins have recently been proposed (6, 7) in the control of gastric acidity and for gastric ulcer therapy. The effect of resin on peptic and tryptic activity being of importance in the biological activity of resins, it was decided to study the problem and to extend the study to cover the physico-chemical behavior of anion exchangers.

### METHOD

The original problem was to determine the effect of Amberlite IR-4 on peptic digestion. When it afterward became apparent that addition of various other surface active agents to Amberlite IR-4 markedly facilitated its dispersal in water, the work was extended to include the effect of several surface agents on peptic digestion.

The *rate* of the process and the *completeness* to which it ultimately proceeds must be carefully distinguished in considering peptic digestion. The initial rate is probably the important factor as far as ulcer therapy is concerned. When various amounts of substances such as Amberlite were added, the final amount of peptic action is very nearly always the same although the rates may have differed markedly. In the literature very little care has been taken to distinguish between capacity and intensity factors. Therefore, the *kinetics* of the peptic digestion of egg albumin have been considered in this paper.

The NF method (using NF reference pepsin) (3400 units/g.) was made suitable for velocity studies by carrying out the reactions in graduated centrifuge vessels which enabled us to read at given intervals of time the amount of egg albumin digested. The rate constants (velocity of reaction) are obtained by determining the limiting slope of the line, time *vs.* concentration. (The density of the egg albumin remains constant throughout if the egg albumin is passed thru a 60-mesh sieve before beginning the reaction.)

The appropriate amount of the material to be studied (*e.g.*, Amberlite, sodium lauryl sulphate, *etc.*) was introduced into the pepsin, dissolved in dilute hydrochloric acid. An aliquot was then introduced into the vessel containing albumin in HCl at 52°C.

## RESULTS

The results are summarized graphically in Figs. 1-5. It will be seen that 0.250 g. of Amberlite IR-4 has no appreciable effect on the rate of hydrolysis (Fig. 3). All results were repeated with the Amberlite IR-4 actually in heterogeneous contact with the reaction solution.

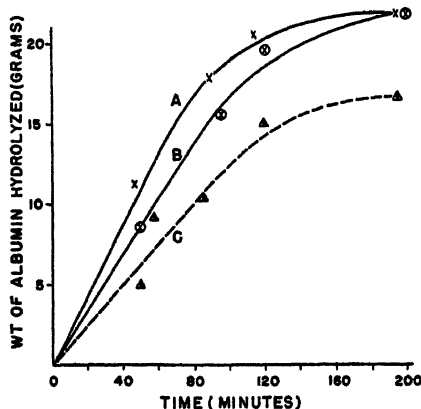


FIG. 1

## Kinetics of Pepsin Hydrolysis

0.5 g. Amberlite IR-4

0.1 g. NF Pepsin

A—Standard ( $m = 1.00$ )

B—Amberlite IR-4 present ( $m = 0.89$ )

C—Amberlite IR-4 filtered off after adsorption ( $m = 0.54$ )

$m$ —slope (given as fractional proportion of the standard)

It will be seen that the rates are different when the Amberlite is actually present in the reaction vessel and when it has been filtered off after being shaken with the pepsin. The rates are greater in the former case. This can be explained by assuming that there is a residual activity of adsorbed pepsin, the action taking place at the surface of the Amberlite IR-4; or by assuming that the pepsin is reversibly adsorbed and a new equilibrium is reached in the reaction vessel proper which liberates a small amount of pepsin.

The rate constant of hydrolysis for albumin in the presence of 0.5 g. of Amberlite IR-4 was 89% (Fig. 1) of the standard. With the resin filtered off the rate constant was only 54% (Fig. 1) of the standard.

Corresponding figures for the system involving 3.0 g. of resin were 39% (Fig. 2) and 17% (Fig. 2). In other words, 3 g. of resin reduce the speed of action of 100 mg. of 3400 Unit pepsin to 39% of its original value. Actual clinical usage of resin in the control of gastric acidity would represent a cross between systems in which the resin remains in contact with the pepsin and systems in which the resin is filtered off.

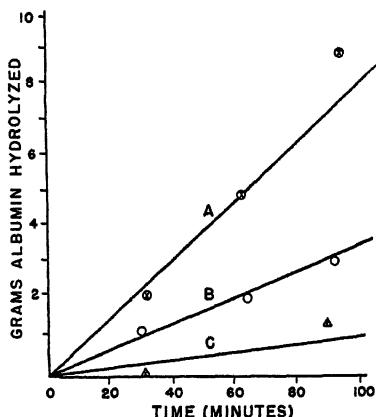


FIG. 2  
Kinetics of Pepsin Hydrolysis

3.0 g. Amberlite IR-4

0.1 g. Pepsin

A—Standard ( $m = 1.00$ )

B—Amberlite IR-4 present ( $m = 0.39$ )

C—Amberlite IR-4 filtered off after adsorption ( $m = 0.17$ )

$m$ —slope (given as fractional proportion of the standard)

Fig. 3 shows the constant difference between the two rates. That is to say, the two plots, "Amount of Amberlite" vs. "Per cent Original Enzyme Activity" are parallel lines.

A concentration of Amberlite less than 0.5 g./0.1 g. pepsin has a negligible effect.

Of surface active agents, Igepon-T (alkyl-CONMeC<sub>2</sub>H<sub>4</sub>SO<sub>3</sub>Na), sulfatate (a hydrocarbon sulfonate) and sodium lauryl sulfate are inhibitory in concentrations upwards of 0.5 g./0.1 g. pepsin, but in very small concentrations (1–2 mg./0.1 g. pepsin) every one of these increases the velocity of the reaction. (See Fig. 4 for sample data on Igepon and sulfatate.)

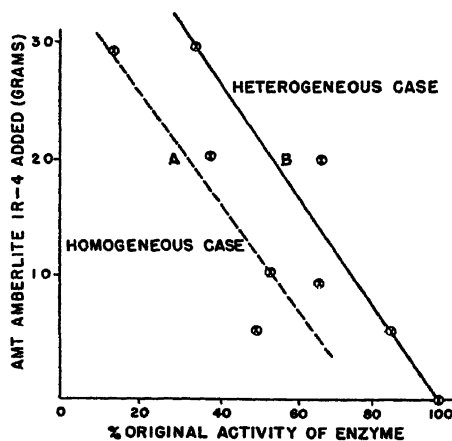


FIG. 3

Effect of Amberlite IR-4 on Activity of Pepsin

A—Homogeneous Case (Amberlite filtered out)

B—Heterogeneous Case ( $G. \text{ Amberlite} = 0.049 (\text{activity of enzyme}) + 4.0$  (Amberlite present))

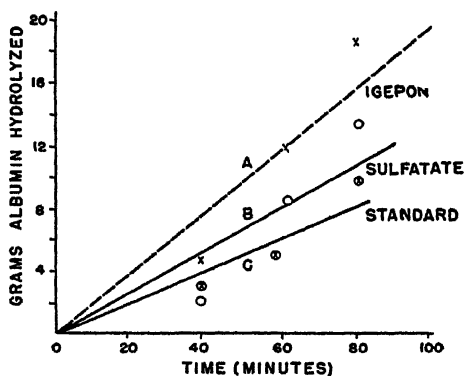


FIG. 4

Effect of Small Amounts of Surface-Active Agents on the Peptic Hydrolysis of Egg Albumin

A—Igepon (2 mg./100 mg. pepsin)

B—Sulfatate (2 mg./100 mg. pepsin)

C—Standard

It is interesting to speculate on the effect of small quantities of materials in the stomach, the presence or absence of which markedly affect the rate of peptic hydrolysis.

For example, large amounts of an agent such as sodium lauryl sulfate would reduce peptic action but, as dilution occurred, as the concentration/unit volume decreased, a point would be reached at which the rate of peptic action would be increased; the overall effect may be an increased activity.

Naturally occurring surface active agents are the various bile acids. The effect of these was tried on the peptic digestion and it was found that small amounts (10 mg. to 0.1 g. pepsin NF) of these materials slow the reaction.

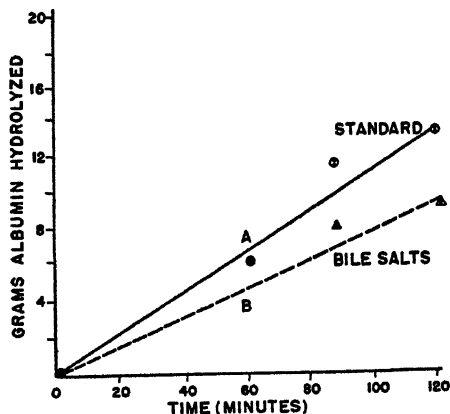


FIG. 5

Effect of Bile Salts on Peptic Digestion

A—Standard

B—Bile Salts (satd. soln. < 10 mg./0.1 g. pepsin)

The mathematical condition of the reaction of the first order is:

$$-\frac{dc}{dt} = kc. \quad (1)$$

The neutralization of hydrochloric acid by Amberlite is certainly not of the first order but, by use of a large excess of one of the constituents (*i.e.*, Amberlite), the concentration of this reactant may be regarded as unchanged during the reaction. The reaction becomes

pseudounimolecular and equation (1) can be used. Temperature and rate of stirring are kept constant throughout.

Integration of equation (1) shows that  $\log (H^+)$  vs. time should be a straight line provided the reaction is truly pseudounimolecular. The experimental data confirm this. Fig. 6 represents the data summarized.

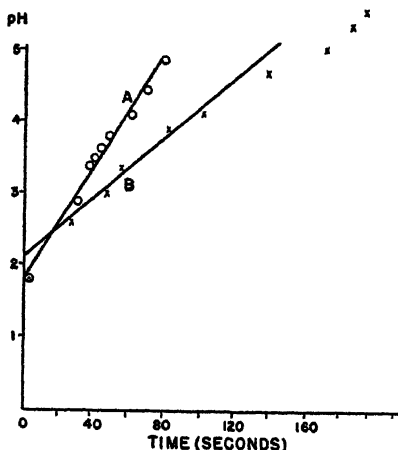


FIG. 6

Rate of Neutralization of Acid by Amberlite IR-4

50 cc. *N*/10 HCl + water

25 g. Amberlite IR-4 (40 mesh)

Speed of Stirring—1120 R.P.M.

A— $k$  36.5° = 0.0370 (pseudomonomolecular)

B— $k$  26.5° = 0.0195 (pseudomonomolecular)

$$k \frac{36.5^\circ}{26.5^\circ} = 1.90$$

$$\frac{d \ln k}{dt} = \frac{E_2}{RT}$$

$$E = \frac{T_2}{T_2 - T_1} \times 2.303 \times R \times \log \frac{k_2}{k_1}$$

$$E = 11,760 \text{ cal.}$$

The velocity constants given represent the limiting slopes of the lines. The temperature coefficient of the reaction between 36.5°C. and 26.5°C. is 1.90. This corresponds to an energy of activation of 11,760 cal.

Amberlite IR-4 shows a pronounced adsorption of sodium chloride and sodium phosphate at a pH of 1.5. This adsorption is "molecular"



and not of the "exchange" type. A Freundlich type isotherm is followed.

Fig. 7 reproduces the results graphically.

The adsorption of these salts is reversible. At the pH of the intestinal tract, (*ca.* 8.5) recovery is apparently quantitative.

At *all* pH's there is no adsorption of NaCl by Amberlite IR-4 from HCl solution.

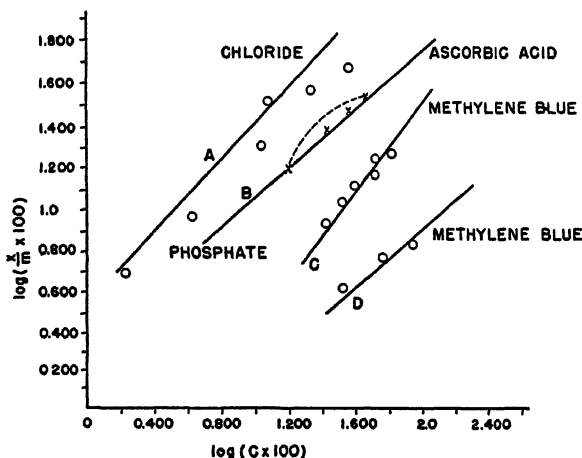


FIG. 7

Adsorption Isotherms for Amberlite IR-4 (from Acid Solution)

- A—Chloride
- B—Phosphate
- C—Methylene Blue (pH = 10.5)
- D—Methylene Blue (pH = 1.5)

The very pronounced adsorption of inorganic ions by Amberlite IR-4 from acid solution is unusual. In general, organic adsorbents adsorb such ions only to a small extent. The further fact that the adsorption manifests a considerable degree of specificity (*e.g.*, chloride ion is adsorbed from nitric acid solution but not from hydrochloric acid solution) naturally leads to the hypothesis that the adsorption of inorganic ions is due to a double salt formation at the interface between the quaternary ammonium salts from the amino groups of the resin and the added salt. It will be remembered that ammonium chloride forms very many such double salts.

The law of combining proportions demands that under such a hypothesis the number of moles of, for example, sodium chloride adsorbed should be proportional to the number of moles of nitric acid adsorbed. Fig. 8, in support of this hypothesis, shows this to be true.

It is apparent that the action of anion exchange resins in heterogeneous systems (resin remaining in the reaction vessel) compares with the action in the gastro-intestinal tract. The total quantity of pepsin in the gastric juice varies between the limits of 3 to 6.5 expressed as mg. of 1 : 4000 USP pepsins (8). Therefore, the indicated dosage of

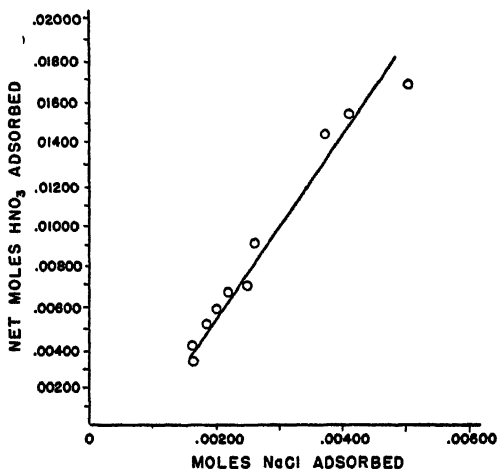


FIG. 8

Proportionality Between Adsorbed Nitrate ( $\text{HNO}_3$ ) and Chloride as Required  
by the Law of Mass Action for the Dissociation of the Postulated  
Double Salt of  $\text{NaCl}$  and Quarternary Ammonium Salt

the anion exchange resin for inhibition of peptic activity would be 100 to 200 mg.

The effect of surface active agents such as sodium lauryl sulfate on the action of pepsin, stimulating in lower concentrations and inhibiting in higher concentrations, doubtless offers an explanation of the discordant results reported in the literature (9, 10, 11) on the effectiveness of surface active agents in the treatment of gastric ulcers. The concentration of the sodium alkyl sulfate present at any given time would determine the ultimate effect. The concentration would vary with dosage and volume of gastric juice present.

The physico-chemical results, disclosing, as they do, marked adsorption of inorganic ions by Amberlite IR-4, suggest that the adsorption of ions is due to double salt formation.

### SUMMARY

The rate of acid neutralization, adsorbent properties and effect on peptic hydrolysis of the synthetic resin Amberlite IR-4 were determined. The reaction rate constants disclosed a reduction of peptic activity to as much as 17% of the original value. The ability of surface agents of the type of sodium lauryl sulfate to speed up peptic action rates in small concentration is reported.

The results indicate the mode of action of resins as gastric antacids and support the hypothesis advanced as to the mechanism of adsorption by resins.

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# Partial Acid Hydrolysates of Proteins. IV. Intravenous Use of High Levels of Fibrin and Casein Hydrolysates in Hypoproteinemic Dogs \*

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## INTRODUCTION

Partial acid hydrolysates of fibrin and casein have been used intravenously in this laboratory to maintain nitrogen balance in dogs (1). Fortification with sulfur-containing amino acids was necessary in the case of casein hydrolysates but not in the case of fibrin hydrolysates. Dogs have been maintained for as long as sixteen weeks on 120 mg. N/kg./day or less of fibrin hydrolysate intravenously (2). The present experiments were designed to test the therapeutic value of partial acid hydrolysates of fibrin and casein at a high level of input under conditions of severe nitrogen depletion. Changes in the plasma protein level were followed and the general effects on the blood picture were observed.

## PROCEDURE

### *Partial Acid Hydrolysates*

The partial acid hydrolysates consisted of mixtures of amino acids and peptides as previously described (3). The casein and fibrin hydrolysates used contained about 25 and 35%, respectively, of free amino acids. The hydrolysates were non-antigenic in repeated tests with guinea pigs and rabbits. No chronic toxicity has been observed in dogs following continuous daily injections for long periods. All preparations used were non-pyrogenic by U.S.P. pyrogen test. The hydrolysates were sterilized by filtration.

Additions of cysteine hydrochloride monohydrate, cystine and dextrose were made as indicated. The additions of amino acids were made in the ratio of 1 mg. of the

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\* A preliminary report of this work was made to the American Society of Biological Chemists in Atlantic City, March, 1946. *Federation Proc.* 5, 134 (1946).

amino acid to 7 mg. of total nitrogen. The protein hydrolysates were made to contain 0.7% nitrogen. The addition of dextrose was 5% W/V.

The essential amino acid composition of the partial acid hydrolysates shown in Table I represents the values obtained from several determinations on successive samples. Determinations were made both by chemical and microbiological methods. Where possible the chemical methods were applied directly to the partial acid hydrolysate, as in the case of tryptophane, cystine, and methionine.<sup>1</sup> For all other determinations, the partial hydrolysate was made 8 N with sulfuric acid and refluxed

TABLE I  
*Essential Amino Acid and Glutamic Acid Content of Partial Acid  
Hydrolysates of Casein and Fibrin*  
Per cent Calculated to 16% N

	Fibrin Hydrolysate				Casein Hydrolysate			
	Chemical Assay		Microbiological Assay		Chemical Assay		Microbiological Assay	
	per cent	Reference	per cent	Reference	per cent	Reference	per cent	Reference
Arginine	6.8	(5)	7.1	(14)	4.1	(5)	4.2	(14)
Histidine	2.5	(4)	2.5	(14)	1.8	(4)	3.4	(14)
Lysine	7.8	(6)	7.8	(14)	5.5	(6)	7.8	(14)
Tryptophane	1.2	(8, 9)	—	—	0.6	(8, 9)	—	—
Phenylalanine	3.5	(4)	3.5	(14)	3.3	(4)	4.0	(14)
Cystine	2.5	(4)	—	—	0.7	(4)	—	—
Methionine	2.0	(10, 11)	2.0	(14)	3.0	(10, 11)	2.9	(14)
Threonine	7.6	(7)	6.8	(14)	4.7	(7)	4.9	(14)
Leucine	—	—	7.0	(12, 15)	—	—	9.4	(12, 15)
Isoleucine	—	—	2.8	(12, 15)	—	—	4.1	(12, 15)
Valine	—	—	6.0	(12, 15)	—	—	7.7	(12, 15)
Glutamic Acid	—	—	15.4	(13, 15)	—	—	27.7	(13, 15)

eight hours to complete the hydrolysis. Methods as compiled by Block and Bolling (4) were used in cases where good recoveries and reproducible results were obtained, as with histidine (Kossel-Kutscher micromodification), phenylalanine (Kapeller-Adler) and cystine (Folin). For arginine most satisfactory recoveries were obtained by the method of Vickery (5) and for lysine by the method of Ayre (6). Threonine was determined by the method of Shinn and Nicolet (7). Tryptophane was determined with good agreement by the methods of Shaw and MacFarlane (8) and Horn and Jones (9). Methionine was determined by the method of McCarthy and Sullivan (10) using the modification suggested by White (11). Leucine, isoleucine and valine <sup>2</sup>

<sup>1</sup> Determinations of tryptophane, methionine and cystine were made by Mr. E. O. Krueger.

<sup>2</sup> The microbiological amino acid determinations were made by Eleanor Willerton.

were determined microbiologically, using the conditions described by McMahan and Snell (12). Threonine, methionine, arginine, histidine, lysine and phenylalanine were determined microbiologically by the method of Stokes *et al.* (14). Check determinations were made in the case of leucine, isoleucine, valine and glutamic acid, using the conditions proposed by Hier *et al.* (15). Glutamic acid was determined by the microbiological method of Dunn *et al.* (13). As a point of interest in this study, glutamic acid was determined directly on both the partial hydrolysates and complete hydrolysates. The content of glutamic acid as determined in the latter was roughly twice that in the former, indicating that only about one-half, or less, of the glutamic acid was available in free form in the partial hydrolysates.

The dried partial acid hydrolysates of fibrin contained, on an average, 14% N and those of casein 13.6% N. Because there was small variation between different lots and because the hydrolysates were used on the basis of their nitrogen content, it was decided to report the amino acid content on a 16% nitrogen basis, as frequently used, rather than on a dry basis.

#### *Non-protein Diet*

A very low nitrogen diet was used for depletion. The diet contained 73 g. sucrose 20 g. lard, 3 g. corn oil, 0.5 g. Haliver oil, 4 g. U.S.P. salt mixture I, 0.2 g. choline chloride, 1 g. agar, 0.6 mg. thiamine hydrochloride, 0.6 mg. riboflavin, 12 mg. nicotinamide, 0.4 mg. pyridoxine hydrochloride and 1.2 mg. calcium pantothenate. Food intake was limited to 80 calories/kg./day. A liver concentrate was used to supply vitamin B<sub>12</sub> ("folic acid") equal to 1.5 g. of 70% alcohol-insoluble liver extract daily. The nitrogen intake on this diet, including vitamin supplements, was 0.05–0.1 g./week.

#### *Analytical Methods*

Plasma protein levels were determined by the method of Barbour and Hamilton (16). The dogs were starved overnight and blood samples taken in the morning. Hemoglobin determinations and red and white blood cell counts were made each week. Total nitrogen determinations were made by macro-Kjeldahl (Arnold-Gunning).

#### *Nitrogen Balance Technique*

Adult female dogs, made parasite-free, were kept in metabolism cages. Provision was made to catch any excreta and vomitus during injection periods. Injections and oral supplements were given on a regular schedule of feeding. The dogs were catheterized and the feces labeled with carmine at the beginning and end of each period. The excreta were made acid, homogenized together in a Waring Blendor and preserved in the cold under toluene prior to sampling.

The dogs were maintained for four to six weeks on the basal diet with fibrin supplement at a level of 100 mg. of nitrogen/kg./day. Following this equilibration period, The dogs were injected at near maintenance levels with fibrin hydrolysate, *i.e.*, 100–120 mg. N/kg./day, to accustom them to daily injections. Nitrogen balance periods on oral fibrin and injection at or near maintenance levels of nitrogen were of 7 days duration.

The dogs were made hypoproteinemic by subjecting them to non-protein feeding. The degree of hypoproteinemia was judged by the plasma protein level, which

generally fell to 4.0–4.5 g./100 cc., or lower, in 4–6 weeks. Nitrogen balances were kept for the last three weekly periods on non-protein diet for each dog. When the degree of hypoproteinemia had become severe, massive injections of fibrin or casein hydrolysates were carried out for 3-day periods. The level of nitrogen given was uniformly 600 mg. N/kg./day, and the rate of injection was 1.5 mg. N/kg./minute.

To arrive at values for the biological value of the hydrolysates under different conditions of injection, it was decided to collect values for "endogenous nitrogen" excretion (EN). Values for excretion on protein-free diet (NE<sub>0</sub>) and values for excretion on maintenance levels of oral fibrin were collected. It was noted that successive periods on protein-free diet gave successively lower values for NE<sub>0</sub>, as previously reported by Seeley (17). The collected values for twenty such periods for five dogs are shown in Table II. The values shown are the limits for each dog. The figures in

TABLE II

*Nitrogen Excretion in Dogs on Non-Protein Diet, on a Maintenance Level of Oral Fibrin, on a Low Level of Injected Fibrin Hydrolysate and on High Levels of Injected Fibrin and Casein Hydrolysates*

Estimates of Endogenous Nitrogen Excretion (En) and Biological Value (BV)

	Input /kg. /day	Nitrogen Excretion/kg./day						BV
	N	Dog 5	Dog 11	Dog 16	Dog 22	Dog 23	Average	
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
Protein-free Diet	0	72–90 <sup>4</sup> (3)	91–109 (5)	86–102 (4)	82–94 (5)	102–108 (3)	88	—
Fibrin, Oral	100	85–101 (3)	—	79–123	90–94 (3)	106–108	100	1
Fibrin Hydrol., i.v.	100	103–126 (6)	106–121	130–161 (6)	121–137	133–165	128	.72
Fibrin Hydrol., i.v.	600	320–461 (6)	306	324–429 (8)	375–427	400–402	380	.53
Casein Hydrol., i.v.	600	500–563 (4)	—	465–565 (5)	—	—	511	.32

\* Maximum and minimum values.

( ) The figures in parentheses show the number of determinations made.

parentheses indicate the number of runs made. The average was 88 mg. N/kg./day. The average of ten values for excretion of the same five dogs receiving 100 mg. N/kg./day in the form of oral fibrin was exactly equal to the intake. Assuming the excretion under these conditions to be equal to endogenous nitrogen excretion as proposed by Mitchell (18), the fibrin would have a biological value of unity. In instances where fibrin was fed orally in maintenance doses to hypoproteinemic dogs, a sparing action on body nitrogen loss has been observed. The biological value for

fibrin calculated for such periods is generally greater than unity and is an example of the situation described by Allison and Anderson (19) in which food nitrogen has a sparing effect on body nitrogen excretion.

For purposes of comparison in Table I, the biological values were calculated from the average nitrogen excretion values. The value for EN was taken as 100 mg. N/kg./day. It should be borne in mind when comparing the biological values resulting from the injection of fibrin hydrolysate at the 100 and 600 mg. nitrogen levels that the rates of injection were 1 and 1.5 mg. N/kg./minute, respectively. On the other hand, the massive doses were given only to hypoproteinemic dogs, whereas the small injection was made in essentially normal dogs.

### *Fibrin vs. Casein Hydrolysates*

Experiments were designed to test the comparative value of the partial acid hydrolysates of fibrin and casein. Since a limiting deficiency of sulfur amino acids had previously been shown to exist in casein hydrolysates, fortification with cysteine hydrochloride monohydrate was made to give a level of cystine plus cysteine equal to the level of cystine shown by analysis to occur naturally in fibrin hydrolysate. By analysis, casein hydrolysate was shown to contain more methionine than fibrin hydrolysate. As the adequacy of sulfur amino acids in fibrin hydrolysate was not known, experiments were conducted both with and without the addition of cysteine to fibrin hydrolysates.

Four adult female dogs which had long been used in nitrogen balance studies at minimum levels of nitrogen, were placed on the above regimen to further deplete their protein stores. One of the dogs died in 7 days on the depletion diet. The remaining dogs fell off in appetite and weight and were brought to an extremely low blood protein level and a state of near inanition in two weeks. Food intake fell nearly to zero during this period. Injections of protein hydrolysates were then begun.

One dog received casein hydrolysate and two dogs received fibrin hydrolysate. The total daily injection volume was about 500–700 cc., depending on the size of the dog. The total time of injection was adjusted to two 3.5 hour periods, morning and afternoon. The level of nitrogen given was 600 mg./kg./day based on the original kennel weight of the dog. The rate of injection was 1.2–1.5 cc. per minute, equal to 1.5 mg. N/kg.

The dog which was started on casein hydrolysate fortified with cysteine became very weak after two days injection. The dog was offered a varied diet but died two days later in a state of inanition.



Dogs 5 and 16 received fibrin hydrolysate with added cysteine for two 3-day periods followed by two more 3-day periods on fibrin hydrolysate with dextrose but no cysteine. To obtain a somewhat comparative test of a casein hydrolysate, dogs 5 and 16 were returned to the nitrogen-low diet for three weeks. Even with this extended depletion, the dogs appeared in a condition much superior to that observed just prior to the use of the fibrin hydrolysate. Therapy was begun again using casein hydrolysate plus dextrose and cysteine, and was conducted for twelve days just as with the fibrin hydrolysate. Occasional vomiting and drooling, particularly during the first hour of

TABLE III  
*Nitrogen Retention and Plasma Protein Regeneration in Protein-Depleted  
Dogs Given Protein Hydrolysates Intravenously*

Days	Regimen	Body wt kg.	Plasma Protein g. /100 cc	Nitro- gen Out- put g.	Nitrogen Balance g	N Re- tained N In- take per cent
Dog 5—Received $13.04 \pm .03$ g. nitrogen during each 3-day injection period.						
1	Non-protein diet	5.54	—	—	—	—
1-6	Non-protein diet	5.54	2.8	3.89	-3.68	—
7-13	Non-protein diet	5.23	—	3.63	-3.47	—
14-16	Fibrin Hyd. + Cysteine <sup>*</sup>	5.12	—	7.99	+4.97	51%
17-19	Fibrin Hyd. + Cysteine <sup>*</sup>	—	3.5	6.88	+6.13	59%
20-22	Fibrin Hyd. + Dextrose	5.54	3.9	8.54	+4.47	46%
23-25	Fibrin Hyd. + Dextrose	5.54	—	9.86	+3.16	36%
26-32	Non-protein diet	5.54	5.6	4.52	-4.22	—
33-39	Non-protein diet	5.68	4.93	4.3	-4.00	—
40-46	Non-protein diet	5.23	4.5	3.69	-3.45	—
47-49	Casein Hyd. + Dextrose + Cysteine <sup>*</sup>	5.12	4.6	10.8	+2.22	29%
50-52	Casein Hyd. + Dextrose + Cysteine <sup>*</sup>	5.12	—	10.5	+2.52	31%
53-55	Casein Hyd. + Dextrose + Cystine <sup>†</sup>	5.12	5.1	11.4	+1.61	24%
56-58	Casein Hyd. + Dextrose + Cystine <sup>†</sup>	5.45	4.8	11.8	+1.23	21%
59-65	Non-protein diet	5.45	4.8	3.99	-3.70	—
66-72	Non-protein diet	5.23	—	4.25	-3.96	—
73-79	Non-protein diet	5.23	4.7	3.58	-3.36	—
80-82	Fibrin Hyd.	5.12	—	8.36	+4.65	48%
83-85	Fibrin Hyd.	5.12	5.5	8.46	+4.61	47%

\* Cysteine hydrochloride monohydrate, 1 mg./7 mg. nitrogen.

† Cystine, 1 mg./7 mg. nitrogen.

TABLE III—*Continued*

Days	Regimen	Body wt. kg.	Plasma Protein g./100 cc.	Nitro- gen (Out- put g.	Nitrogen Balance g.	N Re- tained N In- take per cent
Dog 16—Received $11.14 \pm .03$ g. nitrogen during each 3-day injection period.						
1	Non-protein diet	6.25	—	—	—	—
1-6	Non-protein diet	6.25	3.0	3.74	-3.55	—
7-13	Non-protein diet	6.03	—	3.58	-3.40	—
14-16	Fibrin Hyd. + Cysteine <sup>†</sup>	5.45	—	6.62	+4.52	55%
17-19	Fibrin Hyd. + Cysteine*	—	3.9	5.97	+5.20	60%
20-22	Fibrin Hyd. + Dextrose	6.48	3.7	6.69	+4.48	54%
23-25	Fibrin Hyd. + Dextrose	6.36	—	7.89	+3.27	43%
26-32	Non-protein diet	6.36	5.5	4.84	-4.58	—
33-39	Non-protein diet	6.48	4.7	3.82	-3.52	—
40-46	Non-protein diet	6.25	3.8	4.25	-4.00	—
47-49	Casein Hyd. + Dextrose + Cysteine <sup>†</sup>	6.14	3.9	8.56	+2.60	37%
50-52	Casein Hyd. + Dextrose + Cysteine*	6.36	—	9.08	+2.08	33%
53-55	Casein Hyd. + Dextrose + Cystine <sup>†</sup>	6.36	5.2	9.64	+1.53	28%
56-58	Casein Hyd. + Dextrose + Cystine <sup>†</sup>	6.48	5.0	8.31	+2.84	40%
59-65	Non-protein diet	6.25	4.9	3.75	-3.54	—
66-72	Non-protein diet	6.03	—	4.05	-3.85	—
73-79	Non-protein diet	5.92	4.1	4.01	-3.82	—
80-82	Fibrin Hyd.	5.8	—	7.13	+3.99	50%
83-85	Fibrin Hyd.	—	4.5	6.63	+4.49	55%

injection, occurred with this preparation. The dogs were again placed on the nitrogen-low diet for three weeks and then given fibrin hydrolysate at the same level as above for two more 3-7 day periods.

Nitrogen balance, weight changes, and blood plasma levels during the eighty-five days of successive depletion and nitrogen therapy for dogs 5 and 16 are shown in Table III. Values for hemoglobin and red and white cell counts held relatively constant on the low side of normal throughout and are not shown. Values for nitrogen balance and biologic value are shown. Endogenous nitrogen excretion values were taken as the average of four 3 day periods on protein-free diet. These values were 1.56 g. N for dog 5 and 1.58 g. N for dog 16. If a value of EN of 0.1 g. N/kg./day is used, the biologic values are increased uniformly about 5% for dog 5 and 3% for dog 16.

The appetite response of the dogs, particularly during the periods of fibrin hydrolysate, was dramatic. Voluntary food consumption, which fell off markedly during the depletion periods, was nil by the time injections were begun. After four to six days injection, a progressive increase in appetite occurred until the dogs were taking their full allotment of the non-protein diet.

Since the protein hydrolysate supplied about 20 calories/kg. body weight/day and dextrose, when given, supplied slightly more than this, the dogs received during injection periods about 25–55% more than the 80 calories/kg. minimum ordinarily allowed. Large percentage body weight gains were thus possible and actually did occur, amounting to 10–20% for the first period on fibrin hydrolysate. Voluntary food consumption would probably have been still larger with *ad libitum* feeding.

Further experiments were carried out with dog 16 in which casein and fibrin hydrolysates were supplemented with cysteine at a level of 1 mg./14 mg. N, and methionine at a level of 1 mg./7 mg. N. In successive periods of depletion and massive injection, biological values of 23 and 30% were obtained for the casein hydrolysate and 56% for fibrin hydrolysate. The plasma protein level was below 4.5 g./100 cc. prior to any period of injection.

The constancy of the value for fibrin hydrolysate of about 50–60% utilization under the conditions of massive injection herein described has held true in upwards of 30 individual trials in eight dogs.

#### DISCUSSION

These experiments indicate that much of the nitrogen given to severely depleted animals in the form of a partial acid hydrolysate of fibrin was retained and utilized. Hydrolysates of casein were less well utilized. Preliminary experiments with the partial acid hydrolysate of pancreas residue (Armour) have been even less satisfactory than the experiments with casein hydrolysate at high levels of intake. This may be in part due to the presence of toxic factors in the pancreas residue since these hydrolysates have been shown to cause a pronounced fall in blood pressure on injection in cats.<sup>3</sup> No significant changes in blood pressure were observed following injection of fibrin or casein hydrolysate under similar conditions.

<sup>3</sup> Pharmacologic effects of the various protein hydrolysates have been studied by Dr. R. K. Richards and Dr. G. M. Everett.

The amino acid content of fibrin hydrolysate (Table I) does not appear to be sufficiently superior to that of casein hydrolysate to explain its much greater biological value, particularly when casein hydrolysate is supplemented with cysteine or cystine to an equal or greater level than that in fibrin hydrolysate. Previous work has indicated that sulfur amino acid deficiency is the most important limiting factor for casein hydrolysates, but not for fibrin hydrolysates (1, 2). This is not clearly understandable when it is seen that the combined methionine and cystine contents of the two protein hydrolysates are not widely different. The higher level of tryptophane in fibrin than in casein hydrolysate appears as a basis for difference. However, separate experiments have indicated that tryptophane is not the limiting factor in casein hydrolysates.

The possibility exists that there is a difference in the utilization of peptides as derived from different proteins by the method of partial acid hydrolysis. The possibility also remains that supplementation with cysteine was not at a sufficiently high level in the casein hydrolysates to yield an optimal effect in these experiments. In the previous work of Risser, Schenck and Frost (1) the cystine supplement was made at about twice the level used herein. That deficiency of the sulfur amino acids in casein hydrolysates is clearly the limiting deficiency has been demonstrated both in dogs and in rats. Furthermore, preliminary evidence has appeared which indicates that the methionine in casein hydrolysates is not well utilized. Further experiments are needed to clarify these points.

It is noteworthy that nitrogen retention in most cases reached a peak during the second 3-day period and then gradually fell off. This phenomenon may reflect an improved efficiency of utilization following a brief period of orientation. The decreased retention of nitrogen during the third and fourth periods of fibrin hydrolysate reflects a decreasing avidity for nitrogen, as one would expect. In all, each dog on the first fibrin experiment stored about 18 g. of nitrogen, equal to about 110 g. of dry protein in the 12-day injection period. This represents an increase in total body protein of about 10–13%, considering protein to constitute about 15–20% of body weight.

Elman, Charnas and Davey (20) have suggested the ceiling for nitrogen utilization for nitrogen depleted dogs is above 1.75 g. N/kg./24 hours. Our schedule of 0.6 g. N/kg. in seven hours is fairly comparable

to that of the above authors as regards rate of administration and would allow 2.04 g. N/kg. in 24 hours.

The relative efficiency of nitrogen utilization of any particular preparation, at different rates of injection, remains to be determined. Madden, *et al.* (21, 22) have suggested that the efficiency of utilization of rapidly injected mixtures of amino acids is high in plasma-pherized dogs, but comparison at slow rates was not made and excretion of amino acids was not followed. As hydrolysates injected into the systemic veins must go in large part *via* the kidneys before reaching the liver, there is good possibility of exceeding the renal threshold by too rapid injection. The loss of nitrogen in the form of free amino acids under the conditions described herein was about 8–10% of the nitrogen input, as will be described in a later report. It would seem likely that the more rapid the injection above the threshold level, the more rapid the excretion of amino acids will be.

A further factor in the utilization of mixtures of amino acids and protein hydrolysates on injection may be the tolerance to the injection. As noted above, casein hydrolysate caused frequent vomiting at the rate of 1.5 mg. N/kg./minute. During the 12-day period on casein hydrolysate (Table II) dog 5 vomited only twice, while dog 16 showed reactions on seven of the twelve days and vomited a total of eleven times. No vomiting occurred during the comparable 12-day period on fibrin hydrolysate. In the last 6-day period on fibrin hydrolysate, both dogs vomited several times during the first day of injection, but showed no reaction in the remaining five days.

Several studies have indicated that fibrin hydrolysate is tolerated well at rates greater than 2 mg. N/kg./minute in some dogs but not in others. The maximum tolerated rate for casein hydrolysate appeared to be somewhat lower than that for fibrin hydrolysate. At a rate of 1 mg. N/kg./minute, tolerance to both preparations has been entirely satisfactory. What effect this type of reaction may have on nitrogen utilization in the animal is not known, but it cannot be salutary. Madden *et al.* (23) have reported tolerance studies of this type in considerable detail using both protein hydrolysates and mixtures of pure amino acids, and have indicated that glutamic acid is a prime offender in causing reactions to injection. Unna and Howe (24) have shown that glutamic acid and aspartic acid produce vomiting in dogs when injected in amounts greater than about 200 mg./kg. in a period of

about 1–1.5 hours. It is interesting to note, in light of this disclosure, that fibrin hydrolysate contains less glutamic acid than casein hydrolysate.

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#### SUMMARY

Administration of partial acid hydrolysates of fibrin and casein to protein-depleted dogs by intravenous injection was studied with regard to utilization of the contained nitrogen. 600 mg. N/kg. was given in seven hours each day during repletion periods. The rate of injection was 1.5 mg. N/kg./minute. Nitrogen retained during the first six days of repletion was 50–60% of the nitrogen administered for fibrin hydrolysates and 29–37% for casein hydrolysates. Weight and plasma protein levels were markedly increased following administration of fibrin hydrolysates and were improved to a lesser degree by casein hydrolysates.

The basis for the better utilization of fibrin hydrolysate is not clear, although there is some indication that utilization of methionine as it occurs in the partial acid hydrolysate of casein is limited.

The nitrogen excretion of dogs on non-protein diet averaged 88 mg. N/kg./day. Dogs receiving a maintenance level of 100 mg. N/kg./day in the form of oral fibrin excreted, on an average, exactly this same amount, giving a calculated biological value of 1. Fibrin hydrolysate injected at a level of 100 mg. N/kg./day gave an average biological value of 0.72. Fibrin and casein hydrolysates injected into hypoproteinemic dogs at a level of 600 mg. N/kg./day gave average biological values of 0.53 and 0.32 respectively. The effect of the rate of injection and tolerance to the injected material on utilization is discussed.

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# Certain Factors Affecting Vitamin C Content of Bean Sprouts <sup>1</sup>

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## INTRODUCTION

"To be sure, we cannot have a kitchen garden at sea, and a short and scanty crop of greens can only be raised on board ship; but beans and peas and barley and other seeds brought under the maling process, are converted into the state of a growing plant and if eaten in this state without any sort of preparation, except that of separating or rejecting the husks, cannot fail to supply what is wanted for the cure of scurvy." (Curtis reporting in 1807 on the work of Young (1782) in the British Navy.) It appears to be the first military and naval use of sprouts for specific therapy in scurvy.

During war times a renewed interest has developed (1, 2) in the economic and practical sources of vitamin C in seed sprouts. Earlier investigations (3, 4, 5) have decisively shown that bean and cowpea sprouts contain considerable quantities of ascorbic acid. This finding is probably true for all seed sprouts because, as Bessey and King (6) have pointed out, ascorbic acid is present in greatest concentration in those tissues which are involved in high metabolic activity. The process of ascorbic acid synthesis in plants is further influenced by, and is possibly dependent on, the activity of chlorophyll. Although it is now well recognized that chlorophyll-bearing tissues tend to have the highest concentrations of ascorbic acid, it does not imply a close parallelism (8). Furthermore, wide fluctuations exist in the ascorbic acid content of seedlings (9, 10). These variations in content have been related to the growth of the sprout in light and darkness (11). Thus, Smith and Gillies (7) found a maximum value in potato leaves in the forenoon and minimal values toward the end of night. On the whole, these diurnal variations have not been of great magnitude and do not alter the view that the sprout contains a high concentration of vitamin C.

It is the purpose of this report to present data dealing with these questions, *i.e.*, (a) does the substance which is chemically assayed as

<sup>1</sup> The material in this article should be construed only as the personal opinions of the writers and not as representing the opinion of the Navy Department officially.

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ascorbic acid have equivalent biological antiscorbutic properties; (b) is the concentration of this organic acid always high in sprouts; and (c) does cooking affect the ascorbic acid content of sprouts?

## EXPERIMENTAL

### *Method*

Ascorbic acid and dehydroascorbic acid were assayed in terms of total vitamin C (ascorbic acid and dehydroascorbic acid) and the unoxidized form, by the methods of Bessey (12) and Roe and Keuther (13). All extracting and macerating devices were made of specially cleaned glass or porcelain equipment and every effort was made to obviate contact with metal or other oxidizing agents.

Biological assays were somewhat modified from the usual procedure. The scorbutic diet consisted of casein 10 g., powdered vitamin C-free milk 15 g., boiled alfalfa meal (vitamin C-free) 40 g., brewers' yeast 3 g., and desiccated liver powder (vitamin C-free) 4 g. The diet was moistened, warmed, dried and broken up into fairly large fragments. The fact that guinea pigs consume more food when it is in bulk form has been a common observation in this laboratory and has been recently confirmed by others (14). This is of some importance, since the intake of food bears a relationship to weight gain and survival. The diet used is an artificial one but contained the necessary known food factors beside those specifically required by the guinea pig (15). Preliminary work in this laboratory indicates the presence of a new factor needed by the guinea pig on this artificial diet to approximate the growth curve and weight gain of animals fed diets rich in green vegetables. The guinea pigs used were of a uniform buff strain. The procedure of vitamin assay employed has been adequately described elsewhere (14).

All seeds were sprouted in uniform glassware equipment after an initial soaking of 14 hours. The temperature of the water was maintained at  $20^{\circ}\text{C.} \pm 3^{\circ}\text{C.}$  Ordinary tap water was used. One hundred and fifty g. of beans were placed in a 2,000 ml. chamber and moistened every 10-15 minutes with 400 ml. of water by an automatic self-filling, self-siphoning device.

### *Results*

*Variation and Development of Ascorbic Acid in Bean Sprouts.* Two varieties of soybeans, one variety of mung beans and two of pinto beans were used in this study. The initial soaking was taken as the inception of the sprouting period. The daily increase of ascorbic acid during sprouting was rapid and the variation in final content was considerable (Table I).

Considerable variation of ascorbic acid exists in the raw sprout and exceeds those changes of vitamin content observed in seedlings induced by temperature and diurnal or nocturnal variations (11) and by the addition of sugar to the water used (4). It appears that variation is

intrinsic in the bean since repeated assays from the various samples give approximately the same results. The effect of age of the bean and the condition of the plant producing the bean on the vitamin content have not been studied. As noted by others (4, 5) the epicotyls uniformly contained higher titers of the vitamin than the rest of the bean sprout, which suggests that this is the area of greatest synthesis. (Studies on biosynthesis to be reported later.)

TABLE I  
*Variation in Ascorbic Acid Content in Growing Bean Sprouts*

Days following initial sprouting	mg. vitamin C/100 g. sample or aliquot										
	Soya, Cayuga						Soya, Rich- mond	Mung	Pinto		
	Sample lots in duplicate or triplicate series						Duplicate series	Duplicate series	Sample lots in duplicate or triplicate series		
	I		II		III				I		II
3	8	6	8	3	5	2 1 1	3 2	3 2	6 5 6	7 6	
4	21	13	17	8	9	4 3 5	7 5	8 6	24 16 17	13 14	
5	26	19	20	14	16	5 5 9	14 12	18 15	31 20 21	21 23	
6	24	23	22	16	19	5 6 8	16 16	25 28	42 29 34	28 32	

*Effects of cooking on bean sprouts.*—Various 6-day samples of soya, mung and pinto sprouts were subjected to five minute boiling periods in distilled water. The entire sprout was used and the husks removed by flotation while boiling. In each case 20 g. of beans were used in 100 ml. boiling water (glassware was used throughout). It was found that cooking such sprouts in boiling water brought about a considerable loss of vitamin C (Table II).

*Oxidase from bean sprouts.*—A loss as great as 62.5% of the vitamin in a five minute period of cooking is not likely to be due entirely to the diffusion of the vitamin into the cooking fluid. It seemed probable that some enzyme (oxidase) might be acting on ascorbic acid. To ascertain this, further studies were performed.

Water, specially distilled in glass, was subjected to nitrogen aeration and kept under a layer of oil. Ninety ml. of this water were placed in ten 100 ml. volumetric flasks. In five, the water was immediately covered with a layer of oil and aerated with nitrogen for three minutes. In the other five the water was aerated with air for a

TABLE II

*Loss of Vitamin C (ascorbic acid and dehydroascorbic acid) Due to Diffusion and Oxidation During Cooking (boiling in distilled water 5 minutes)*

mg. vitamin C/100 g. 6-day bean sprouts				
Bean	Sample	Before cooking	After cooking	Per cent loss
Soya Cayuga	1	20	12	40.0
	2	23	14	39.0
	3	22	11	50.0
	4	16	7	56.3
	5	19	13	31.2
Richmond	1	16	7	56.3
	2	16	6	62.5
Mung	1	25	15	40.0
	2	28	19	32.1
Pinto	1	29	14	51.7
	2	34	13	61.8
	3	28	11	60.7
	4	32	17	46.9

similar period. Thus, five of the flasks contained aerated water and five contained water relatively free of oxygen. To each of these flasks 20 mg. of ascorbic acid (C.P.) were added. The flasks were then paired and the following substances tested for oxidase activity: (a) raw bean cotyledons and stems, (b) raw husks, (c) cooked bean cotyledons and stems, (d) cooking fluid from cooked beans (20 g. beans in 100 ml. water), and (e) the control of ascorbic acid following aeration with air and nitrogen. The material was prepared by grinding 5 g. in a mortar and taking up the juice in 20 ml. distilled water (specially prepared). Each volumetric flask was then filled to the 100 ml. volume with the aqueous extract to be tested. In the case of the control the volume was made up with water. The entire procedure was done fairly rapidly so that the materials could be tested simultaneously under the same experimental conditions. An initial 10 ml. aliquot was taken for assay and similar aliquots at 10 and 20 minute intervals. Active oxidation of ascorbic acid to dehydroascorbic acid took place only with the uncooked cotyledon and stem extract, the other samples having very little if any enzymatic action (Table III).

The results show that the oxidase action appears to be primarily concerned with the first step of aerobic oxidation of ascorbic acid to dehydroascorbic acid and results in an eventual loss of dehydroascorbic acid beyond diketogulonic acid to, presumably, threonic and oxalic

acids. For example, 1.74 mg. of ascorbic acid in the 10 ml. samples changed concentration to 0.57 mg. of ascorbic acid and 0.6 mg. of dehydroascorbic acid. The sum of these two (1.17 mg.), however, represents the total vitamin C content, and shows a loss of 32.8% of the original value. The high content of dehydroascorbic acid is indicative of active enzymatic action.

TABLE III

*Oxidation of Ascorbic Acid by Various Portions of Soya (Cayuga Bean Sprout Extracts)*

Mg. vitamin C in 10 ml. sample							Per cent oxidation	
Position	Condition	Ascorbic acid			Dehydroascorbic acid		Ascorbic acid	Actual total loss
		Initial value	10 min.	20 min.	Initial value	Terminal value		
Cotyledons Stems, raw	aerobic	1.74	1.16	0.57	negligible	0.6	67.2	32.8
	anaerobic	1.65	1.63	1.35	trace	0.13	18.1	10.0
Husks	aerobic	1.86	1.63	1.53	negligible	0.18	18.1	8.0
	anaerobic	1.86	1.86	1.53	trace	0.19	18.1	7.5
Cooked beans	aerobic	1.86	1.86	1.63	negligible	0.10	12.4	7.0
	anaerobic	1.86	1.86	1.53	trace	0.09	18.1	13.0
Fluid from cooked beans	aerobic	1.86	1.86	1.63	negligible	0.12	12.4	6.0
	anaerobic	1.86	1.74	1.63	trace	0.09	12.4	7.2
Ascorbic acid control	aerobic	2.07	1.86	1.74	negligible	0.08	16.0	12.0
	anaerobic	1.86	1.86	1.74	trace	0.05	6.4	4.0

As the sprout extracts are derived from macerated samples, it would seem unlikely that such a high degree of enzymatic action would occur with the whole bean sprout during ordinary cooking procedures. For this reason a 10 g. sample of the same bean sprouts (soya-Cayuga) was placed in 100 ml. of boiling distilled water and boiled for five minutes. The beans were then separated from the water by a strainer. Aliquots from a gross sample were assayed previous to cooking. Following the boiling the entire 10 g. and cooking fluid were analyzed for their vitamin

C content. By this method it was possible to ascertain the loss of the vitamin by the ordinary cooking method of boiling (Table IV).

TABLE IV  
*Vitamin C Loss in Bean Sprouts by a 5 Minute Boiling Period*

	Mg ascorbic acid in substance assayed		
	(a) Raw sprouts	(b) Cooked sprouts	(c) Cooking fluid
Dehydroascorbic acid	none	0.1	0.4
Ascorbic acid	1.6	0.61	0.2
Total vitamin C	1.6	0.71	0.6

Loss of vitamin from sprout into cooking water = (a) - (b) = 0.89 mg. (55.3%).  
Loss of vitamin by oxidation = (a) - (b) + (c) = 0.29 (18%).

The total loss of the vitamin from the sprout during cooking is 55.3%, but 38% of this loss can be accounted for by its presence in the cooking liquor. The loss by oxidation, whether in the bean sprout proper or in the liquor has a significant value of 18%. The total loss compares quite well with those observed in Table II.

*Biological assay of vitamin C in cooked sprouts.*—It appears that from 0.75–1.2 mg. of ascorbic acid, daily, seem to satisfy the requirements of the weanling guinea pig for this vitamin. For assay, five groups of 10 pedigreed buff strain weanling pigs were used. All of the groups were placed on the scorbutic diet. One group remained on this diet, the others received the following daily supplements: 0.5 mg. ascorbic acid per pig; 1.2 mg. ascorbic acid per pig; 0.5 mg. ascorbic acid present in cooked sprouts per pig; 1.2 mg. ascorbic acid present in cooked sprouts per pig. Growth curves gave equivalent comparable results for the guinea pigs receiving the vitamin in synthetic form and those receiving it in a natural form. Both groups on 0.5 mg. of the vitamin daily exhibited a poor weight gain; those on 1.2 mg. made the expected gain and those animals on a vitamin C-free diet died at varying intervals of 18–26 days and were autopsied. Adrenal, liver, bone marrow and muscle were sectioned for study; the presence of hemorrhages and the defect in collagen and reticulum were regarded as pathognomonic for scurvy. The others were killed in 30 days for similar study and failed to exhibit scorbutic lesions.

## ACKNOWLEDGEMENT

Acknowledgment is made to A. Wetterstrom, PhM2c, V10, USNR, for assistance, and to Lt. Commander C. M. McCay, H(S), USNR for advice.

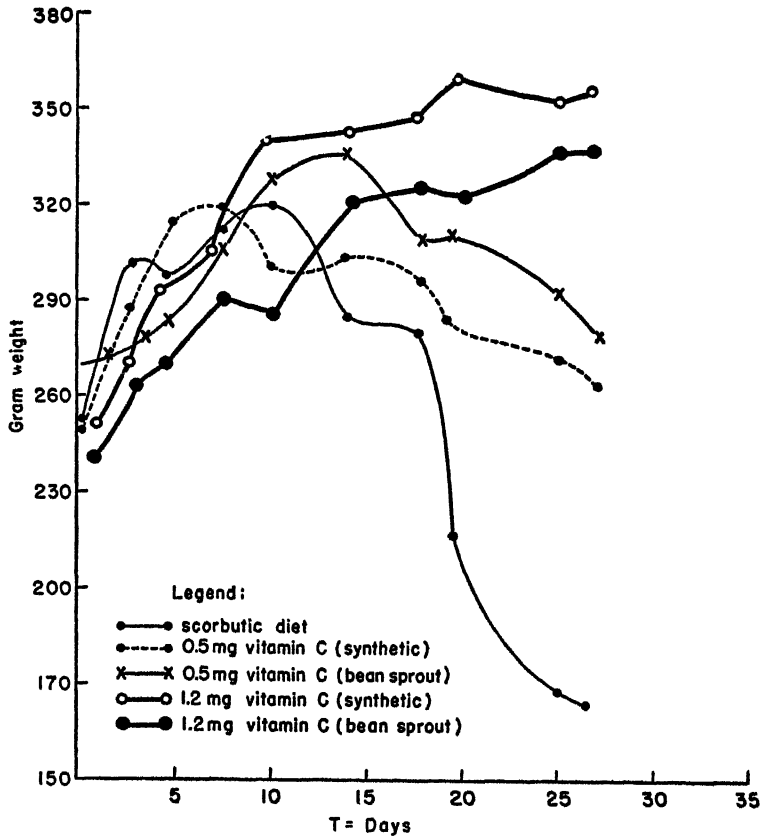


FIG. 1

Average Growth Curves of Guinea Pigs (20 to each series) receiving Vitamin C from Synthetic and Natural Sources

## SUMMARY

1. Bean sprouts may lose some vitamin C (56%) during cooking but still remain a fair source of the vitamin. The amount present in

cooked sprouts (6-19 mg. *per cent*) is comparable to that found in certain potatoes and cabbages.

2. The loss of vitamin C in bean sprouts through processing is due in part to its diffusion into cooking fluid and in part to enzymatic oxidation.

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# The Structure of the Water-Insoluble Pigment of the Bark of "Sangre de Drago"

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## INTRODUCTION

The bark of the "Sangre de Drago" tree contains an interesting coloring material. Although the color of the bark is red, on extraction with hot water another coloring material is removed, and the bark thereby assumes a deeper red color than it possessed prior to this extraction. It is this second water-insoluble red pigment with which this investigation is concerned. The "Sangre de Drago" species grows abundantly in a territory of Mexico which is in the provinces of Michoacan and Guerrero.

## EXPERIMENTAL

### *Isolation of the Pigment*

About 30 kg. of bark were exhaustively extracted with boiling water until the water drained off colorless. The dried residue was macerated with benzene in the cold for 7 days to remove resinous material. The pigment was then extracted with 30 liters of petroleum ether. All but 500 ml. of the solvent was removed *in vacuo* whereby a yellow solid material settled out. This was filtered off and 1 liter of water added to the filtrate. The residual petroleum ether was removed *in vacuo*, when the red pigment settled out because of its insolubility in water. The product was filtered off, washed with water and dried. The crude material (29 g.) was recrystallized several times from methanol until a constant m.p., 292°C. was obtained.

Anal.: C, 76.4; H, 6.2; N, 7.3

The simplest possible over all formula seems to be:



### *Bromination of the Pigment*

One g. of the red substance, dissolved in petroleum ether, was treated with a solution of bromine in  $\text{CCl}_4$  until bromine was no longer absorbed. No  $\text{HBr}$  was given off and the uptake of bromine amounted to 0.43 g. On the basis of the molecular



weight of the parent compound (718), one molecule would take up 320.78 g. or two molecules of bromine. The analysis of the brominated product showed this to be the case.

The solvent was removed *in vacuo* and the crude brominated compound recrystallized from methanol.

Anal.: Calc. for  $C_{16}H_{16}O_4N_1Br_1:Br$ , 30.8

Found for  $C_{16}H_{16}O_4N_1Br_1:Br$ , 29.9

This substance has, accordingly, two unsaturated double bonds in the molecule.

#### *Hydrolysis of the Pigment*

Five g. of pigment were hydrolyzed with 200 ml. of  $H_2SO_4$  at pH 4 for 16 hrs. The reaction mixture was neutralized with  $BaCO_3$  and then extracted with chloroform. Evaporation of this solution *in vacuo* gave rise to 2.329 g. of a hydrolysis product, A, which was recrystallized from ethanol. The white needles so obtained melted at 209°C.

Anal.: C, 70.1; H, 6.0; N, 14.6

The chloroform-extracted residue was thereupon extracted with ether and, on evaporation of the ether *in vacuo*, a second hydrolysis product, B, was obtained (2.32 g.). It was a pink crystalline substance which, on recrystallization from ethanol, melted at 217°C.

Anal.: C, 76; H, 6.6

#### *Bromination of Compound B*

The bromination of compound B was carried out as previously described. One half g. of the product took up 0.42 g. of bromine. The brominated compound was isolated and recrystallized from ethanol.

Anal.: Calc. for  $C_{24}H_{26}O_4Br_1:Br$ , 45.8

Found for  $C_{24}H_{26}O_4Br_1:Br$ , 44.9

#### *Ozonization of Compound B*

Three g. of the compound were treated with ozone according to the method of Harries (1) and the ozonide formed decomposed by treating with water. A solid fraction obtained was filtered off and recrystallized from ether; m.p., 114°C.

Anal.: Calc. for  $C_4H_6O_8:C$ , 26.3; H, 3.3

Found for  $C_4H_6O_8:C$ , 26.2; H, 3.1

#### *Preparation of Derivatives of n-Propylphenyl ketone*

The oxime and a semicarbazone of the liquid fraction obtained from the hydrolysis of the ozonide of compound B were prepared according to standard procedures. The m.p. of the oxime was 49.9°C. and of the semicarbazone 183.7°C.

#### *Solid Fraction Obtained from Hydrolysis of the Ozonide of Compound B*

The solid derivative obtained as a result of the hydrolysis of the ozonide of compound B on recrystallization from ether melted at 114°C. These data would identify

this compound as being dihydroxytartaric acid, since the melting point and analysis correspond satisfactorily.

Anal.: Calc. for  $C_4H_6O_8$ : C, 26.3; H, 3.3

Found C, 26.2; H, 3.1

*Preparation of the Diethyl Ester of Compound B*

The diethyl ester of compound B was prepared according to the method of Groggins (2), 0.5 g. of the compound and about 10 g. of ethyl alcohol being used.  $CaCl_2$  was added, the excess of alcohol removed *in vacuo* and the solution extracted with ether. The solvent was then removed *in vacuo* and the residue recrystallized from ethanol; m.p., 244°C.

Anal.: Calc. for  $C_{28}H_{34}O_4$ : C, 77.4; H, 7.8

Found for  $C_{28}H_{34}O_4$ : C, 76.9; H, 7.6

*Ozonization of the Diethyl Ester of Compound B*

The method of Harries was again employed to ozonize the diethyl ester. The crude product obtained was then hydrolyzed with water. One of the compounds present, *n*-propylphenylketone, was distilled (219.5°C.). The residue of the distillate was taken up with ethanol and, after removal of the latter *in vacuo*, the residue distilled again. B.p., 233°C.

Anal.: Calc.  $C_8H_{10}O_6$ : C, 47.5; H, 4.9

Found  $C_8H_{10}O_6$ : C, 47.3; H, 4.5

## DISCUSSION

The pigment obtained by petroleum ether extraction of the bark, following an exhaustive extraction with hot water and benzene, is ruby red in color, crystalline and melts at 292°C. It is soluble in petroleum ether or hot methanol, slightly soluble in cold methanol and insoluble in water. Microqualitative analysis of the compound established the presence of C, H and N and the absence of S and halogens. Micro analysis established the following quantitative relation of the elements present:

C, 76.4; H, 6.2; N, 7.3.

Accordingly, the simplest empirical formula would be  $C_{46}H_{46}O_4N_4$ . This is corroborated by the molecular weight of the compound determined according to the Rast method. The calculated molecular weight is 718 and the value found is 714.3.

The presence of two unsaturated carbon linkages in the molecule was established by the fact that two molecules of bromine were taken up by the compound without evolution of HBr. On hydrolysis of the pigment two products were obtained. Component A is soluble in

chloroform and, on recrystallization from ethanol as white needles, melts at 209°C. and is laevorotatory  $[\alpha]_D^{14} = -254^\circ$  in  $\text{CHCl}_3$ . Component B was obtained by ether extraction of the chloroform-extracted residue. It is a pink crystalline material which, on recrystallization from ethanol, melts at 217°C.

Micro qualitative analysis of component A established the presence of C, H, N and the absence of S and halogens. The following quantitative relations of the elements present was established by micro analysis:

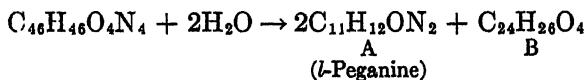
C, 70.1; H, 6.0; N, 14.6

The simplest empirical formula would be, therefore,  $\text{C}_{11}\text{H}_{12}\text{ON}_2$ . The molecular weight of the compound, determined according to the Rast method, is 185.7 agreeing with a theoretical empirical formula of  $\text{C}_{11}\text{H}_{12}\text{ON}_2$ .

*l*-Peganine is described in the literature as having the empirical formula  $\text{C}_{11}\text{H}_{12}\text{ON}_2$ , being laevorotatory  $[\alpha]_D^{14} = -254^\circ$  in  $\text{CHCl}_3$  and melting at 210°C. The data for component A correspond with the data for *l*-peganine thus establishing its identity.

Similarly, the following data were obtained for component B. Presence of C and H by micro analysis: C, 76; H, 6.6. The simple empirical formula, therefore, would be  $\text{C}_{24}\text{H}_{26}\text{O}_4$ . This is corroborated by molecular weight determination. The value obtained is 375.9 while the theoretical value is 378 for  $\text{C}_{24}\text{H}_{26}\text{O}_4$ .

There are two hydrolysis products of the parent compound  $\text{C}_{46}\text{H}_{46}\text{O}_4\text{N}_4$ , the formation of which could be visualized as follows:



The latter compound is acidic in nature and can be titrated with sodium hydroxide using phenolphthalein as indicator. There is indication that the two unsaturated linkages found in the parent compound are part of compound B. It takes up two molecules of bromine giving rise to a tetrabromo derivative. On the basis of analytical evidence the formula for the newly formed product is  $\text{C}_{24}\text{H}_{26}\text{O}_4\text{Br}_4$ .

Advantage was taken of the unsaturated nature of compound B by treating it with ozone forming an ozonide. Upon treating the ozonide with water, two products were obtained, one liquid and the

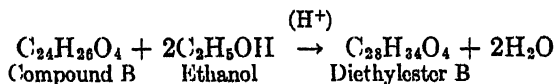
other solid. The liquid portion was purified by distillation, boiling at 219.5°C. Qualitative examination of this compound indicated the presence of a carbonyl group by reacting with phenylhydrazine and dinitrophenylhydrazine. Accordingly, the oxime of the compound, m.p. 49.9°C., as well as the semicarbazone, m.p. 183.7°C., was prepared. A search of the literature reveals that these data agree well with those available for *n*-propylphenylketone, establishing the nature of one component of compound B, the empirical formula being  $C_{10}H_{12}O$ .

The solid product obtained from the ozonide of compound B on treatment with water, was recrystallized from ether and found to have a m.p. of 114°C. Assuming that on splitting an ozonide with water two breakdown products are obtained, both containing carbonyl groups, one should expect this compound to possess such a group. This, however, was not the case. Possibly such a product is affected by the conditions of the reaction giving rise to another compound after passing through the stage of the carbonyl compound. The following analytical data for the compound melting at 114°C. prove the presence of C and H; C, 26.2; H, 3.1.

These data correspond with those presented in the literature for dihydroxytartaric acid: m.p. 114°C; C, 26.3; H, 3.3.  $C_4H_6O_8$ .

The identity of the compound being established as dihydroxytartaric acid, its precursor formed during the splitting of compound B with water and containing a carbonyl group could be diketosuccinic acid. Data in the literature corroborate the ease with which this compound gives rise to dihydroxytartaric acid on treatment with water.

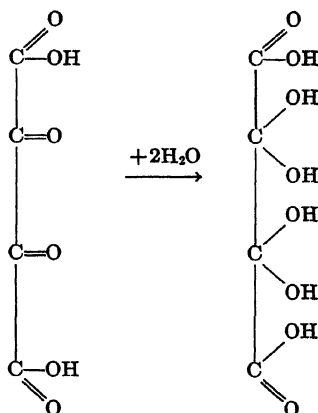
Additional proof for the formation of diketosuccinic acid is afforded by the preparation of the diethyl ester of compound B, treatment with ozone and splitting of the ozonide



to give the diethyl ester of diketosuccinic acid and *n*-propylphenylketone.

The diethyl ester of compound B analyzed as follows:

Calc. for  $C_{28}H_{34}O_4$ : C, 77.4; H, 7.8  
 Found for  $C_{28}H_{34}O_4$ : C, 76.9; H, 7.6



Diketosuccinic acid      Dihydroxytartaric acid

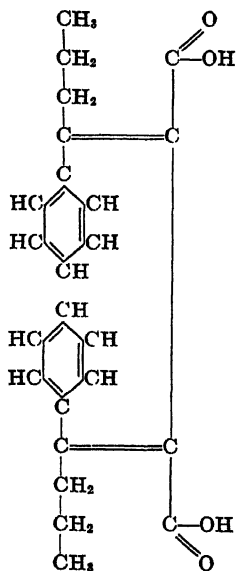
The diethyl ester of diketosuccinic acid,  $C_8H_{10}O_6$ , analyzed as follows:

Calc. for  $C_8H_{10}O_6$ : C, 47.5; H, 4.9

Found for  $C_8H_{10}O_6$ : C, 47.3; H, 4.5

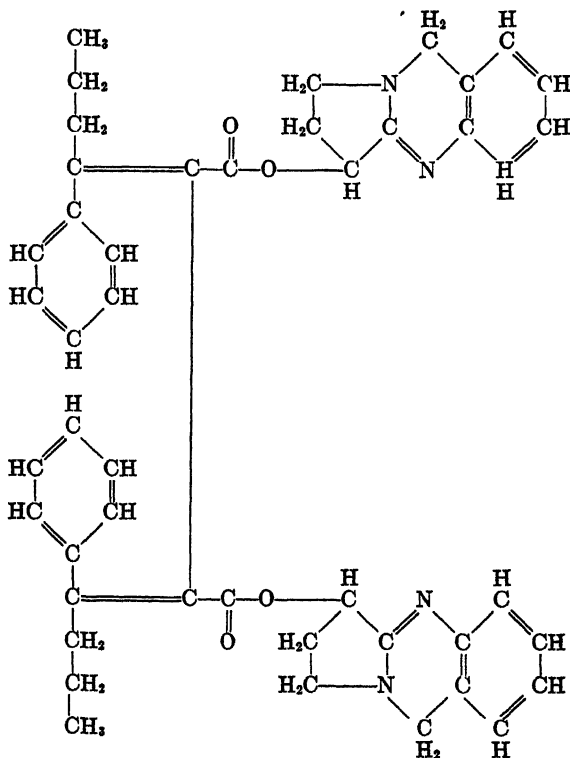
and boiling at  $233^\circ\text{C}$ . These data correspond with those available in the literature for the diethyl ester of diketosuccinic acid.

Considering the data presented, compound B could be the result of union of two molecules of *n*-propylphenylketone and one molecule of diketosuccinic acid:



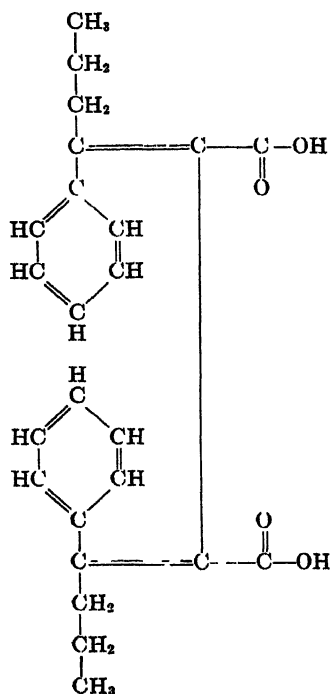
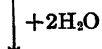
Such a compound would be di(*n*-propylphenyl)-fulginic acid.

The following structure is, accordingly, proposed as corresponding with the qualitative and quantitative properties of the pigment. The schematic presentation of the degradation involved follows:

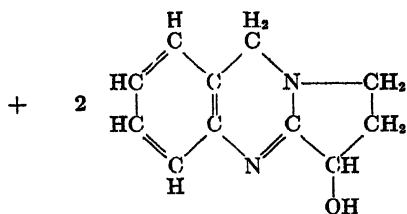


NORDINE

Red Pigment  $C_{46}H_{46}O_4N_4$



Di(*n*-propylphenyl)-fulginic acid  
 $C_{24}H_{26}O_4$   
 B

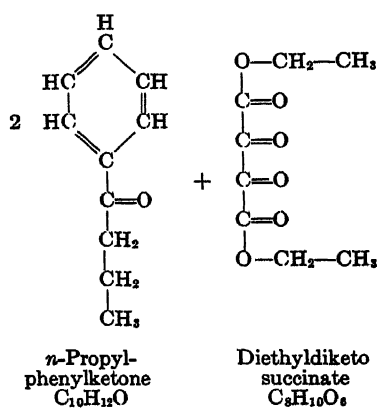
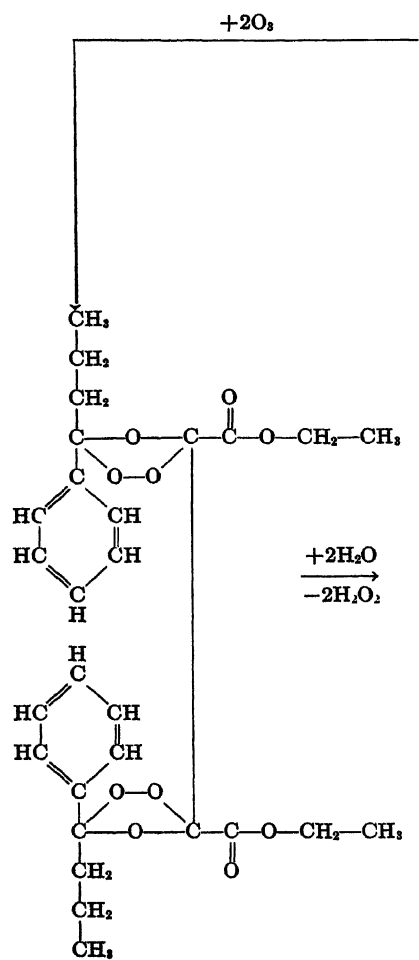
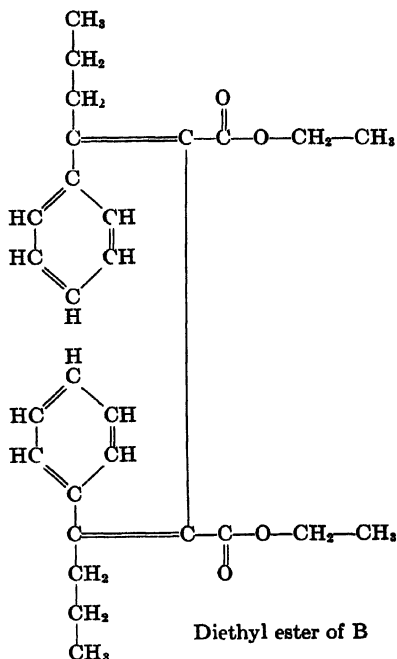
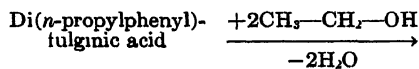


*l*-Peganine  
 $C_{11}H_{12}ON_2$

A







## ACKNOWLEDGMENT

I wish to express my sincere appreciation to Mr. James C. Vitucci of the Department of Organic Chemistry, Fordham University, New York, for assistance in preparing this manuscript.

## SUMMARY

The red coloring material extracted from the "Sangre de Drago" tree has been shown to have the empirical formula  $C_{46}H_{46}O_4N_4$ .

It has been shown to be the product of the union of two molecules of *l*-peganine,  $C_{11}H_{12}ON_2$ , and one molecule of di(*n*-propylphenyl)-fulginic acid. Accordingly, the pigment should be chemically designated as the di(*l*-peganine ester) of di(*n*-propylphenyl)-fulginic acid.

It is proposed to name this pigment: Nordine.

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# Effect of Alkaloids on Acetylcholine Synthesis\*

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## INTRODUCTION

It is known that some of the recognized alkaloids have a specific stimulator or inhibitor effect on nerve tissue and on the different effector organs. It is currently held that acetylcholine is synthesized in nerve tissue, is released by cholinergic nerves and participates in the induction of response of effector cells to indirect stimulation. The purpose of the following investigation was to determine: (1) whether certain alkaloids modify the synthesis of acetylcholine; and (2) whether the difference in the known pharmacological effects of the alkaloids can be explained on the basis of their action on the synthesis of acetylcholine.

## METHOD

The effect of the alkaloids on the acetylcholine synthesis was studied by the method described previously (1, 2). Mixtures containing varying amounts of the alkaloids used (pH corrected to 7.4), 100 mg. of minced fresh frog brain, 3 mg. of physostigmine salicylate and 3 cc. of Ringer's solution were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized *rectus abdominis* muscle of the frog. Whether the alkaloids modified the sensitivity of the *rectus abdominis* to the acetylcholine content of the mixtures during the 2 minutes of immersion for the biological assay was also ascertained by adding the substances to incubated control mixtures after incubation. If the alkaloids modified the sensitivity of the *rectus abdominis* to acetylcholine, the changes were accounted for in the calculation.

*Calculation*—The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical non-incubated mixtures. The amount of acetylcholine synthesized

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\* This study was aided by a grant from the John and Mary R. Markle Foundation.

in the control mixtures containing only brain, physostigmine and Ringer's solution was taken as 100%. The acetylcholine content of the mixtures containing the various substances used was expressed as a percentage of the control.

## RESULTS

The amount of acetylcholine synthesized in the presence of the alkaloids used are given in Table I. In higher concentrations (3 mg./

TABLE I

*Effect of Alkaloids on Acetylcholine Synthesis*

Substance	Amount of acetylcholine synthesized in per cent of control*					
	Amounts (in mg.) of the substances added to 100 mg. frog brain:					
	3	0.3	0.03	0.003	0.0003	0.00003
Amphetamine Sulfate	51	75	90	105	106	—
Atropine†	—	66	103	97	99	—
Cinchonine	8	58	107	106	105	—
Cocaine	31	92	120	126	130	—
Codeine	76	93	97	101	98	—
Colchicine	14	65	81	106	103	—
Ephedrine	64	112	127	127	130	—
Ergotamine	—	111	122	126	137	108
Morphine	65	90	99	99	101	—
Pilocarpine	69	96	96	103	100	—
Quinidine	4	43	85	109	104	—
Quinine	4	31	70	99	102	100
Strychnine	6	42	90	102	103	—
<i>d</i> -Tubocurarine†	—	45	104	104	105	—
Veratrine†	—	29	64	81	102	—
Yohimbine	70	107	100	97	103	—

\* Each value represents the average of 10 separate experiments. The S.E. of the mean for each value was less than  $\pm 5\%$ .

† Atropine, *d*-tubocurarine, and veratrine were not used in higher concentrations since in those concentrations they prevent acetylcholine from acting on the test object, the sensitized *rectus abdominis* muscle of the frog.

100 mg. frog brain) all the alkaloids used decreased the acetylcholine synthesis.

In lower concentrations cocaine, ephedrine and ergotamine increased the synthesis of acetylcholine. [Epinephrine is known to increase the acetylcholine synthesis in concentrations from  $1 \times 10^{-8}$  to  $1 \times 10^{-6}$

mol. from 50–150% (3).] Yohimbine and pilocarpine, in lower concentrations, did not modify the acetylcholine synthesis.

The other alkaloids, in lower concentrations, decreased the acetylcholine synthesis; veratrin decreased the acetylcholine synthesis even in very low concentrations.

### DISCUSSION

In higher concentrations (3 mg./100 mg. frog brain) the alkaloids studied decreased the acetylcholine synthesis. This decrease was probably due to some physicochemical property common to all alkaloids, *e.g.*, denaturation of proteins. Free —OH groups alone do not duplicate the decrease of acetylcholine synthesis found in the presence of alkaloids, since the enzyme preparation synthesizes more acetylcholine at alkaline pH than at acid pH. Chemically related compounds (ammonia, pyrrole, pyridine, piperidine, quinoline) are also known to decrease acetylcholine synthesis (4, 5, 6) if used in higher concentrations.

The effect of epinephrine in increasing the synthesis of acetylcholine may offer an explanation of the observation that epinephrine increases the effect of acetylcholine in the central nervous system (7).

Some organs are more sensitive to atropine and others are more sensitive to curare. The present experiments suggest that such difference in action of these alkaloids is not due to their effect on acetylcholine synthesis since similar concentrations of atropine and *d*-tubocurarine were required to comparably decrease the synthesis of acetylcholine.

Strychnine did not increase the synthesis of acetylcholine. These results suggest that the effect of strychnine on the central nervous system is not due to increased acetylcholine synthesis.

The authors wish to express their gratitude to Dr. A. R. McIntyre for the generous supply of *d*-tubocurarine, to Smith, Kline and French Laboratories for the amphetamine sulfate, and to Sandoz Chemical Works, Inc., for the ergotamine.

### SUMMARY

1. The effect of 16 alkaloids on the synthesis of acetylcholine was investigated by a modified method of Quastel, Tennenbaum and Wheatley.

2. All the alkaloids used decreased the acetylcholine synthesis in a concentration of 3 mg./100 mg. frog brain. Epinephrine, cocaine, ephedrine and ergotamine, in lower concentrations, increased the acetylcholine synthesis by from 20–150%. Yohimbine and pilocarpine, in lower concentrations, did not modify acetylcholine synthesis. Veratrine, quinine, quinidine, strychnine, amphetamine sulfate, morphine, codeine, cinchonine, colchicine, *d*-tubocurarine and atropine decreased the synthesis in lower concentrations.

3. The specific stimulator and inhibitor effect of some of the alkaloids used on the nervous system and effector organs seem to be independent of their effect on the synthesis of acetylcholine.

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# The Distribution and Comparative Content of Certain B-Complex Vitamins in Chicken Muscles

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## INTRODUCTION

It has been shown previously that the various muscles from a single animal may differ markedly in their content of certain of the B-complex vitamins (1, 2, 3, 4). Also, it has been observed that the concentration of each vitamin is correlated to some extent with that of the other vitamins, *e.g.*, pork muscles rich in thiamine usually contain high levels of niacin but relatively low levels of riboflavin and pantothenic acid (4). Other workers have shown that the thiamine, riboflavin and pantothenic acid content of dark chicken meat is greater than that of the light meat (5, 6, 7, 8, 9). On the other hand, the niacin content of the dark meat has been reported to be lower than that of the light (9, 10).

These variations in vitamin content must be related in some manner with the metabolism of the different muscles. However, with the data now available regarding the distribution of vitamins in individual muscles and the metabolic functions of the vitamins it is difficult to explain the specific relationships between vitamin content and muscle metabolism. By comparing the vitamin content of several muscles of one species with the vitamin content of the same muscles of other species it may be possible to obtain additional information which will assist in elucidating the role of vitamins in muscle. Accordingly, a study has been made of the distribution of vitamins in chicken muscles as representing avian tissue. This species seemed particularly interesting for study since variations in the colors of the muscles are quite apparent.

## EXPERIMENTAL

Thirteen skeletal muscles, the heart, the liver, the gizzard and a portion of the skin from each of three White Rock chickens were used in the vitamin studies. The first



chicken, a cockerel, and the second, a pullet, were approximately four months old. These birds had been raised on practical poultry rations supplying approximately 2.4  $\gamma$  thiamine, 1.9  $\gamma$  riboflavin, 26.8  $\gamma$  niacin and 5.5  $\gamma$  of pantothenic acid/g. of feed. The third chicken was an 11 month old White Rock pullet from a laying flock. While this bird was not actively laying, immature eggs were present in the ovary. The mash being used as feed for the laying flock at this time contained approximately 2.77  $\gamma$  thiamine, 14.5  $\gamma$  riboflavin, 28.1  $\gamma$  niacin, and 5.6  $\gamma$  of pantothenic acid/g.

The muscles from two young birds (same flock as the first two) were used for the proximate analyses as some of the muscles were too small to supply sufficient tissue for both vitamin and proximate analyses. Another bird from this flock was used for determination of the relative intensities of the colors of the muscles.

Each bird was killed by piercing the brain and severing the jugular veins, allowed to bleed thoroughly, defeathered and then dissected immediately. All surface fat and as much of the tendons and fascia as possible were removed from the muscles. For the vitamin analyses, paired muscles from the two sides of each bird were pooled, weighed, cut into small pieces, covered with water and allowed to stand for a few minutes. Using a Beckman Model G pH meter the pH value of each sample was determined. The sample was then washed into a Waring Blendor and blended with sufficient water to give one to three dilution, including the water previously used to moisten the sample.

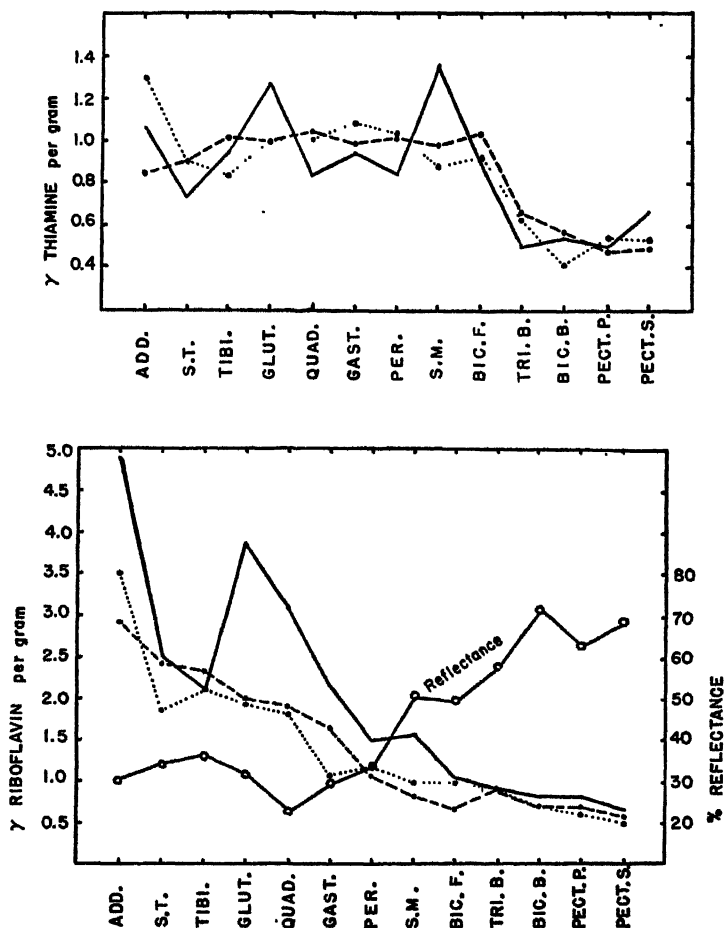
Portions of the blends were weighed into flasks for extraction of the vitamins according to the procedure of Cheldelin *et al.* (11), with the exception that the enzyme-treated suspensions were heated in a boiling water bath for only 10 minutes. It has been shown that longer treatment results in significant losses of thiamine (12). Thiamine analyses were conducted directly on the filtered solutions, using the thiochrome procedure of Hennessy and Cerecedo (13). Riboflavin determinations were made according to the fluorometric method of Peterson, Brady and Shaw (14). Also, microbiological riboflavin assays were conducted by a method similar to that of Snell and Strong (15). Niacin and pantothenic acid analyses were made microbiologically on dilutions of the filtered extracts (16 and 17).

Because of the small size of some of the muscles, only one analysis of each sample was made for each vitamin (except in cases where the values seemed atypical). However, use of these methods on similar series of analyses indicates that duplicate analyses would probably have checked those shown within 10%. Likewise, because of the small amounts of samples, the various muscles from the two birds taken for proximate analysis were pooled to give sufficient tissue for use in the application of the Association of Official Agricultural Chemists' methods for protein and moisture.

Since the color of muscles is largely due to various respiratory pigments, intensities of the colors of the muscles were estimated using a Photovolt reflectance meter, Model 610, and a blue filter of the tri-stimulus series. To increase sensitivity the instrument was adjusted to show 100 scale deflections using a grey color card which showed 29% reflectance, based on 74% for the standard glazed white plate. Each muscle was slit longitudinally to show the color of the interior of the muscle and pressed flat under a microscope slide to obtain a plane surface before reflectance readings were taken. Attempts were made to select areas free from fat and connective tissue, either of which would give a large increase in reflectance. Most of the muscles

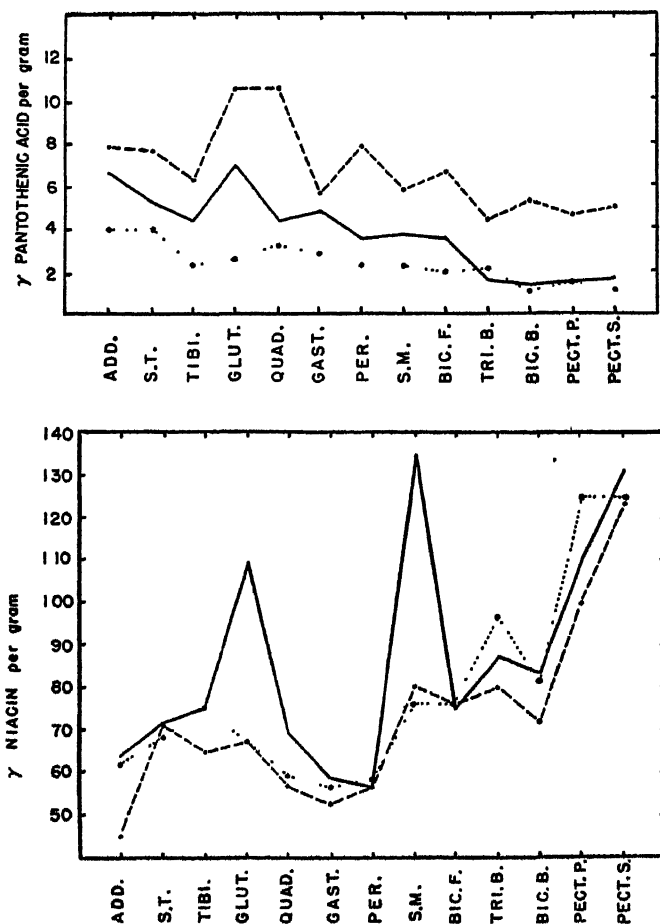
were fairly uniform in color, but several were noted in which there was considerable variation. In such cases an area representing average reflectance was selected. Reflectance readings with a single filter include variations in both hue and intensity, but for purposes of correlations with vitamin content it was thought unnecessary to obtain values for total reflectance.

The data obtained have been arranged in Tables I and II and Figures 1-5 to permit comparison of the vitamin contents of the various muscles and to show the correlations discussed below.



## DISCUSSION

The vitamin content of chicken meat differs markedly from one muscle to another, even in the same bird. These variations are in agree-



FIGS. 1-4

Variations in Vitamin Content of Chicken Muscles

Figures 1-4 ..... Chicken No. 1, — Chicken No. 2, ----- Chicken No. 3. In Fig. 2, o—o traces the reflectance values, (see text) for the muscles of a chicken from the same flock as Nos. 1 and 2. The muscles have been arranged arbitrarily along the abscissa in order of descending value of riboflavin.

ment with the results obtained by other investigators studying light and dark chicken meat (5, 6, 7, 8, 9, 10), but they further indicate great differences between "dark" muscles. By inspection of the data shown in Table I and Figs. 1-4 it can be noted that all of the birds

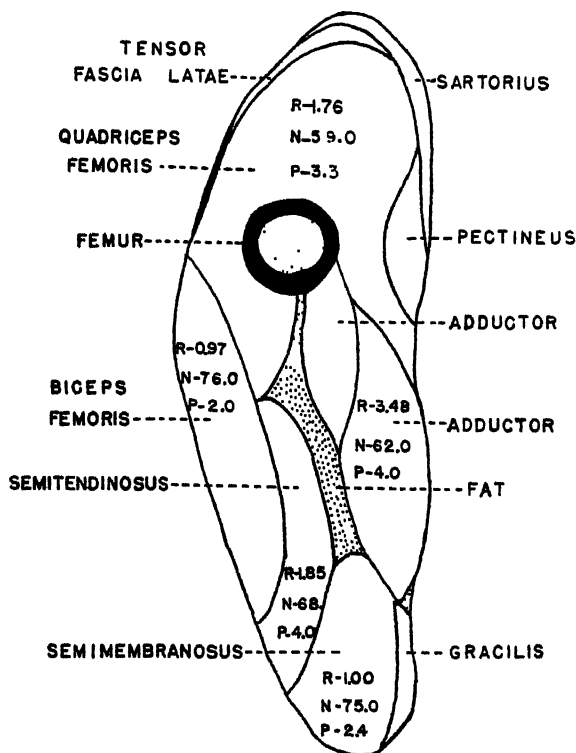


Fig. 5

Cross Section of the Thigh of a Chicken Showing Positions of the Muscle and Vitamin Contents as Found for Bird No. 1. The numbers preceded by an R represent the riboflavin concentration in γ/g. Likewise P and N denote pantothenic acid and niacin values.

showed approximately the same vitamin distribution pattern, *i.e.*, a muscle which was high in a vitamin in one chicken tended to be high in the same vitamin in the other chickens.

While the thiamine contents of the darker muscles are two to three times those of the lighter muscles, the differences between the various

TABLE I

Sample*	$\gamma$ of Vitamins/Gi. of Tissue											
	Thiamine			Riboflavin**			Niacin			Pantothenic Acid		
	Animal No.			Animal No.			Animal No.			Animal No.		
	1	2	3	1	2	3	1	2	3	1	2	3
Biceps femoris	0.9	0.9	1.0	1.0	1.2	0.7	75.6	75.5	75.0	2.0	3.6	6.7
Semitendinosus	.9	.8	.9	1.9	2.5	2.5	68.5	72.5	71.5	4.0	5.3	7.6
Quadriceps femoris	1.0	.8	1.0	1.8	3.1	2.0	59.0	69.5	57.0	3.3	4.4	10.0
Gluteus group	1.0	1.3	1.0	1.9	3.8	2.1	60.8	108.5	87.0	2.6	7.0	10.0
Semimembranosus	.9	1.4	1.0	1.0	1.0	.8	75.6	135.5	80.0	2.4	3.8	5.8
Adductor	1.3	1.1	.8	3.5	4.9	2.9	61.8	65.0	45.0	4.0	6.6	7.8
Gastrocnemius	1.1	1.0	1.0	1.1	2.2	1.7	50.8	58.5	53.5	2.9	4.8	5.7
Peroneus longus	1.0	.8	1.0	1.2	1.6	1.1	58.3	56.5	56.5	2.4	3.6	7.9
Tibialis cranialis	.8	.9	1.0	2.1	2.1	2.2	75.0	75.5	65.5	2.4	4.2	6.3
Pectoralis superficialis	.5	.7	.5	0.5	0.7	.7	124.8	131.7	123.7	1.2	1.6	4.9
Pectoralis profundus	.5	.5	.5	0.7	0.8	.7	125.0	110.0	100.0	1.5	1.5	4.6
Biceps brachii	.4	.5	.6	.7	.8	.7	82.0	84.0	72.0	1.1	1.3	5.4
Triceps brachii	.6	.5	.7	1.0	.9	1.0	96.8	86.5	80.0	2.2	1.6	4.4
Liver	.9	.6	.6	21.7	29.4	15.4	139.0	118.0	137.5	22.0	17.0	27.5
Heart	2.2	2.1	.9	10.5	9.6	8.3	29.1	77.5	45.6	12.6	11.5	12.5
Gizzard	.4	.4	.3	2.1	2.4	2.0	45.6	53.0	40.7	2.8	3.0	3.4
Skin	.1	.2	.1	.9	1.3	0.4	16.3	35.0	7.0	1.2	2.0	1.5
Ratio highest muscle to lowest	3.1	2.7	2.1	6.6	6.9	2.2	2.2	2.4	2.7	3.6	5.4	2.4

\* Nomenclature of muscles based on that of Chamberlain (18).

\*\* The values shown were obtained fluorometrically but microbiological analyses gave essentially the same figures.

dark muscles (or the various light muscles) are not sufficiently great to clearly define a distribution pattern within those two conventional classifications (Fig. 1).

With riboflavin, however, greater differences exist between muscles. Not only do the dark muscles show two to five times more riboflavin than the light muscles, but certain of the dark muscles are outstanding. Thus, in all three birds the adductor contains the most riboflavin and the semitendinosus, quadriceps femoris, gluteus and tibialis cranialis muscles tend to be significantly higher than the biceps femoris, semimembranosus, gastrocnemius and peroneus longus. Relationships such as that between the biceps femoris and the semitendinosus are especially interesting, since these muscles lie side by side, yet differ in riboflavin content by 100% or more (Fig. 5). Likewise, the semimembranosus, one of the dark muscles lowest in riboflavin, lies between the adductor and semitendinosus, both of which are rich in this vitamin. The data shown were obtained fluorometrically, but exactly the same picture was obtained with microbiological analyses.

Although the data are less consistent, the general pattern with respect to pantothenic acid is similar to that for riboflavin (Fig. 3). Again the dark muscles are richer than the light. The adductor, gluteus, quadriceps femoris and semitendinosus muscles are richest in pantothenic acid, and the biceps femoris, semimembranosus and peroneus longus are among the dark muscles low in pantothenic acid. As with riboflavin, muscles of low and high potencies lie side by side. It is interesting to note that the pantothenic acid values for bird No. 3 are considerably higher than those for birds No. 1 and No. 2, regardless of the fact that the feeds contained similar amounts of this vitamin. Whether the higher pantothenic acid content observed for this bird is typical of older or laying birds was not determined.

Although the differences in potencies of niacin are not so marked as those for the other vitamins, they show interesting trends (Fig. 4). With respect to this vitamin the light muscles are definitely richer than the dark muscles. The adductor, which is rich in riboflavin and pantothenic acid, is one of the poorest in niacin. Similar inverse relationships tend to hold with the other muscles.

Excepting thiamine, the above relationships parallel those noted in the pig (4). There it was observed that muscles rich in riboflavin were usually rich in pantothenic acid, but that they were usually poor in thiamine and niacin. In the chicken, thiamine seems to parallel the riboflavin and pantothenic acid values rather than the niacin. Perhaps the peculiar ability of the pig to accumulate thiamine in its tissues will eventually be shown to be connected with these differences.

The pH values for the light muscles are definitely lower than those for the dark muscles but there is little variation within the dark muscles (Table II). Hence this factor does not seem directly related to vitamin content. Muscle size, as indicated by weight, also seems independent of vitamin potency. Likewise, variation in proximate analyses do not explain the fluctuations in vitamin values.

That the colors of the muscles may be correlated with vitamin contents is evident from Fig. 2, in which the reflectometer readings are plotted along with the riboflavin values. In general, the dark muscles contained the largest amounts of thiamine, riboflavin and pantothenic acid and the lowest amounts of niacin. In the chicken these darker muscles, being located in the legs and back, undoubtedly have greater functional activity than the wing and breast muscles. These observa-

TABLE II

Sample	Weight in g.*		pH		Color*†	Wa- ter**	Pro- tein†‡	Fat***
	No. 1	No. 3	No. 1	No. 3				
						<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Biceps femoris	27.7	37.0	6.0	6.2	50	76.0	22.1	1.8
Semitendinosus	18.5	18.2	6.0	6.2	34	77.6	19.9	2.5
Quadriceps femoris	29.4	40.5	6.0	6.4	23	76.5	21.2	2.3
Gluteus group	21.1	22.0	6.1	6.3	32	76.5	21.1	2.4
Semimembranosus	14.4	21.9	6.1	6.1	51	76.8	21.8	1.4
Adductor	15.5	18.8	6.1	6.3	30	76.6	20.1	3.3
Gastrocnemius	47.7	53.6	6.1	6.3	32***	76.1	21.8	2.1
Peroneus longus	15.2	15.1	6.1	6.3	34	75.2	22.9	2.6
Tibialis cranialis	15.3	17.4	6.0	6.3	36	77.8	20.9	1.3
Pectoralis superficialis	125.1	189.9	5.7	5.6	69	73.4	24.5	2.1
Pectoralis profundus	49.7	83.4	5.7	5.6	63	74.3	24.1	1.6
Biceps brachii	6.7	8.6	5.8	5.7	72	75.0	21.9	3.1
Triceps brachii	14.8	19.4	6.1	6.1	58	75.6	22.4	2.0
Liver	36.9	35.0	6.0	6.2	—	73.2	17.8	9.1
Heart	5.0	4.0	6.6	6.3	—	78.2	15.7	6.1
Gizzard	48.7	31.8	6.6	6.4	—	76.8	18.8	4.4
Skin	—	—	6.3	6.8	—	69.5	20.4	10.1

\* Weight of muscles from both sides of chickens No. 1 and No. 3.

\*\* See text. These values represent chickens from the same flock as No. 1.

\*\*\* Portions of this muscle were much lighter, but the reading shown seemed most typical.

tions agree with those of Palladin (19) who reports "the greater the work capacity of muscles . . . the greater is its flavine content." Although differences in intensity of color of the "dark" muscles could not be correlated with vitamin content in every case, it is significant to note that, in all chickens examined, the semimembranosus and biceps femoris muscles were noticeably lighter in color than the other "dark" muscles and that their vitamin contents were intermediate between the "light" and "dark" muscles. This lends support to the assumption that color, function and vitamin content are interrelated.

Gross examination of the color of ground pork muscles revealed no consistent relationship between color and vitamin content (4). Failure to observe better correlation may have been due to the use of ground muscles in which tendon and fat contributed to the general color. Even there, however, those muscles showing definitely higher ribo-

flavin and pantothenic acid values tended to be dark in color as compared to those low in these vitamins. In this connection it might be pointed out that the relatively light colored inactive pork loin contained low amounts of riboflavin and pantothenic acid but higher amounts of thiamine and niacin, thus resembling chicken breast muscle with the exception of thiamine.

The available evidence thus indicates that in both species color, function and vitamin content may be interrelated. This might well be expected in view of the participation of both vitamins and muscle pigments in cellular metabolism. It is hoped that further investigation will more clearly define these relationships and assist in elucidating the roles of the various active components.

### SUMMARY

Thiamine, riboflavin, niacin and pantothenic acid have been determined in thirteen skeletal muscles, the livers, hearts and gizzards of three chickens.

The vitamin contents of muscles from a single bird vary as much as 500%, typical values being, in terms of  $\gamma/g.$  of muscle: thiamine, 0.4–1.3; riboflavin, 0.5–3.5; niacin, 56.8–125.0; and pantothenic acid, 1.1–4.0. Vitamin contents of the gizzards also fall within these ranges. While the hearts and livers do not differ significantly from the muscles in thiamine or niacin content, they do contain more riboflavin, the ranges being 9.3–10.5 and 15.5–29.4  $\gamma/g.$ , respectively. Likewise, the pantothenic acid content of these organs is high, being 11.5–12.6  $\gamma/g.$  of heart and 17.6–27.5  $\gamma/g.$  of liver.

Muscles which are rich in a vitamin in one bird tend to be high in the same vitamin in other birds. There appears, therefore, to be a general vitamin distribution pattern for the relative amounts of the vitamins in the various muscles. Muscles containing relatively much thiamine usually contain high levels of riboflavin and pantothenic acid, but relatively low levels of niacin.

Although the vitamin contents of the "dark" muscles differ widely among the various "dark" muscles from each bird, these tissues contain two to three times more thiamine, riboflavin, and pantothenic acid than the lighter colored muscles. The reverse is true with niacin, however, the "light" muscles being two to three times richer in this vitamin.



Inasmuch as those muscles rich in thiamine, riboflavin and pantothenic acid tend to be darkest in color and to be the most active, it is probable that color, vitamin content and function of muscles are interrelated.

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# Biological Inactivity of $\zeta$ -Carotene <sup>1</sup>

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## INTRODUCTION

Recently Porter and Zscheile (1) found considerable quantities of  $\zeta$ -carotene in certain experimental tomato strains.<sup>4</sup> The extraction, isolation and purification of this pigment has been described by Nash and Zscheile (2), who also presented the specific absorption curve of the new carotene. This paper reports the bioassay of  $\zeta$ -carotene in comparison with  $\beta$ -carotene for vitamin A activity.

## EXPERIMENTAL

### *Method of Preparing Solutions*

$\zeta$ -Carotene was chromatographed on magnesia <sup>5</sup>—Super-Cel and alumina columns until free from all colored impurities. It was eluted with alcohol and hexane and, after removal of the former, the purity and quantity of  $\zeta$ -carotene were determined spectroscopically. The desired quantity of hexane solution was pipetted into Wesson oil having a peroxide number of approximately 4, and 0.3% of  $\alpha$ -tocopherol was added. The hexane was removed from the oil at room temperature under reduced pressure. The remaining solution was poured into several test tubes. These were then sealed under vacuum and stored (approximately 2 months) at  $-20^{\circ}\text{C}$ . until the biological assays were made.

The  $\beta$ -carotene used as a standard for biological comparison was obtained from

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<sup>2</sup> Ensign U.S.N.R. This article is not intended to reflect official views of the Navy Department.

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<sup>4</sup> [Pan American  $\times$  F<sub>1</sub> (Pan American  $\times$  *L. hirsutum* 892)]  $\times$  *L. hirsutum* 892 = Federal Plant Introduction No. 126446.

<sup>5</sup> No. 2641 Adsorptive Magnesia, Westvaco Chlorine Products Co., Newark, Calif.

tomatoes. After extraction, it was chromatographed on magnesia-Super Cel columns until free from other carotenes. It was crystallized from hexane and ethanol (9). Some of the carotene was then dissolved in hexane and its purity determined spectroscopically. The remaining steps in the preparation of samples were the same as for  $\zeta$ -carotene solutions. Only one solution of  $\beta$ -carotene was prepared (0.3  $\gamma$ /drop).

#### Method of Assay<sup>6</sup>

The U.S.P. method of assay as outlined in the U.S.P. XII was used.

Twelve rats were used on each level of carotene. They were fed from calibrated droppers at the following levels.

$\zeta$ -carotene	1.2 $\gamma$ /day
$\zeta$ -carotene	10.0 $\gamma$ /day

U.S.P. Reference Cod Liver Oil No. 3 1.5 U.S.P. units Vit. A/day, calculated equivalent to 0.9  $\gamma$  of  $\beta$ -carotene

$\beta$ -carotene	0.6 $\gamma$ /day
$\beta$ -carotene	0.9 $\gamma$ /day
$\beta$ -carotene	1.2 $\gamma$ /day

The samples were kept at 4°C. during the assay period. No samples were opened until required for feeding purposes and then at least two fresh tubes were used per week. Table I shows that the  $\beta$ - and  $\zeta$ -carotene solutions were stable under these conditions for at least 4 days after opening the ampoules.<sup>7</sup>

TABLE I  
*Stability of Carotene Solutions Used in Bio-assays*

Wave length Å	$\beta$ -Carotene Absorption $\left(\log \frac{I_0}{I}\right)$ in hexane at		
	0 days	2 days	6 days
4780	.392	.393	.394
4500	.450	.447	.449
4360	.349	.351	.352
	$\zeta$ -Carotene		
	0 days	4 days	
4250	.480	.482	
4000	.498	.484	
3860	.252	.253	

<sup>6</sup> The bioassays were performed under the supervision of Dr. C. H. Krieger, Wisconsin Alumni Research Foundation Laboratory, Madison, Wisconsin.

<sup>7</sup> Aliquots were dissolved in 25 ml. hexane and then extracted successively with two 25 ml. portions of 20% KOH in methanol, and two 25 ml. portions of 90% methanol. The methanol solutions were re-extracted with a small quantity of hexane. The combined hexane solutions were washed, filtered and made to volume.

## RESULTS

$\zeta$ -Carotene showed no vitamin A activity at either level of feeding. Of the twenty-four rats fed at the two levels, only seven survived the four week assay period. Only two of these gained weight (3 and 5 g.).

The rats on the three levels of  $\beta$ -carotene far exceeded the expected increase in weight during the assay period. Table II shows the growth

TABLE II

*Comparison of Growth Response of Vitamin A Deficient Rats Fed  $\beta$ -Carotene,  $\zeta$ -Carotene and Reference Cod Liver Oil No. 3*

Supplement	Gain in Weight* 28 days gms.
$\beta$ -Carotene	
0.6 $\gamma$ daily	35.6
0.9 $\gamma$ daily	51.2
1.2 $\gamma$ daily	53.3
Reference Cod Liver Oil No. 3 (1.5 U.S.P. units daily)†	32.2
$\zeta$ -Carotene	
1.2 $\gamma$ daily	— 25.0 (only 3 survived)
10.0 $\gamma$ daily	— 10.0 (only 4 survived)

\* Average for 12 rats.

† Calculated equivalent to 0.9  $\gamma$  of  $\beta$ -carotene.

of the rats on the three levels of  $\beta$ -carotene, two levels of  $\zeta$ -carotene, and on the one level of U.S.P. Reference Cod Liver Oil.

## DISCUSSION

Of the carotenes occurring in largest amounts in most fruits and vegetables only lycopene and its isomers lack vitamin A activity.  $\alpha$ -,  $\beta$ -, and  $\gamma$ -carotenes and all of their *cis*-isomers, which have been tested, have some activity (3). The lack of activity shown by  $\zeta$ -carotene plus the similarity of the shape of its light absorption curve to that of lycopene strongly suggests that the two have the same general type of structural configuration.

Recently, Deuel *et al.* (4) noted that vitamin A deficient rats fed pure  $\beta$ -carotene gained much more weight during an assay period than animals fed an equal number of units (calculated) of vitamin A in

U.S.P. Reference Cod Liver Oil.<sup>8</sup> The same results were obtained in these assays. Table III shows that our data on  $\beta$ -carotene check well with those of the California workers. The basal diet used in the assay of our materials contained ample tocopherol; experiments had shown that further additions of  $\alpha$ -tocopherol did not increase the growth

TABLE III

*Summary of Growth of Rats fed Reference Cod Liver Oil No. 3 and  $\beta$ -Carotene Prepared at Purdue University and California Institute of Technology*

Supplement	Dose per day	Number of rats completing assay	Av. wt. prior to depletion	Av. depletion period	Average body weight during assay period (28 days)		
					Initial	Final	Change
	$\gamma$		g.	days	g.	g.	g.
$\beta$ -carotene <sup>1</sup>	0.6	11 <sup>2</sup>	41.0	32.5	92.4	128.0	35.6
$\beta$ -carotene <sup>3</sup> (5)	0.6	15	41.5	24.0	88.8	114.0	25.2
$\beta$ -carotene <sup>3</sup> (3)	0.6	16	41.8	21.0	84.0	113.4	29.4
$\beta$ -carotene <sup>1</sup>	0.9	12	41.1	32.6	93.0	144.2	51.2
$\beta$ -carotene <sup>3</sup> (4)	0.9	16	43.4	18.4	75.4	129.1	53.7
Reference C.L.O. No. 3							
1.5 U.S.P. units <sup>4</sup>		12	41.7	32.6	93.4	125.6	32.2
$\beta$ -carotene <sup>1</sup>	1.2	12	40.9	32.6	93.8	147.1	53.3
$\beta$ -carotene <sup>3</sup> (5)	1.2	16	42.0	22.0	91.2	139.1	47.9
$\beta$ -carotene <sup>3</sup> (3)	1.2	15	43.0	19.0	86.8	138.4	51.6

<sup>1</sup> Prepared at Purdue University; assayed at Wis. Alumni Res. Found., Madison, Wis.

<sup>2</sup> Only male animals were used in our assays, while the California group used both males and females.

<sup>3</sup> Prepared at California Institute of Technology; assayed at University of Southern California.

<sup>4</sup> Calculated equivalent to 0.9  $\gamma$  of  $\beta$ -carotene.

response to cod liver oil (8). Accordingly it would appear that the U.S.P. reference standard actually contains less vitamin A units/g. than it is stated to contain. If so, many data obtained by biological assays need re-evaluation before they can be compared quantitatively with chemical data (4, 6, 7).

<sup>8</sup> After this paper was complete, a comparison of the vitamin A activity of U.S.P. Reference Cod Liver Oil No. 2 and crystalline  $\beta$ -carotene was reported by Callison and Orent-Keiles (10). These workers obtained results very similar to those found in our laboratory and by the California workers. Rats fed 1.0  $\gamma$  of  $\beta$ -carotene daily gained 53.2 g. in 28 days.

## ACKNOWLEDGEMENT

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## SUMMARY

$\zeta$ -Carotene was assayed for vitamin A activity and found inactive.

The rats fed pure  $\beta$ -carotene gained much more weight during the assay period than expected. This result is discussed in relation to the present vitamin A standard.

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# Some Properties of a Flour Proteinase

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## INTRODUCTION

The existence of proteolytic enzymes in wheat flour is well known to biochemists, and has been noted by Balland (1884), and measured by Ford and Guthrie (1908) and Swanson and Tague (1916). Baker and Hulton (1908) and Stockham (1920) also found proteolytic activity to be present in flour by adding flour to suitable substrates and determining changes in the substrate induced by the proteinase contained in the flour. Cairns and Bailey (1928) obtained a ferment preparation from aqueous flour extracts that digested casein. Various other workers have also contributed to our knowledge of flour proteinases.

Balls and Hale (1936, 1936a, 1938) described the preparation and properties of a proteinase from wheat bran, and thought it was essentially papain-like in type, since it resembled papain in several of its characteristics. Hale (1939) also extracted a proteinase from patent flour, without, however, satisfactorily purifying it. The behavior of this preparation toward reducing and oxidizing agents led Hale to conclude that it contained an enzyme of the papain type and similar to the proteinase extracted from wheat bran. Olcott, Sapirstein and Blish (1943) found a flour proteinase adsorbed on gluten dispersed in acetic acid to be responsible for the changes suffered by gluten in this reagent (Rose and Cook, 1935; Cook and Rose, 1935). This proteinase was inactivated by heating for short periods at 100°C. and no changes in the physico-chemical properties of the dispersion were detected on standing. Harris and Johnson (1945) confirmed the presence of proteinase in acetic acid dispersions of gluten and the inhibitory effect of heat.

The presence of active proteinase in acetic acid dispersions of gluten offered an opportunity to observe certain properties of the enzyme without the necessity of preparing it in the dry, purified form. As pointed out by Balls and Hale (1938), there are distinct advantages in working with purified preparations since the concentration and purity can be well controlled, and more direct comparisons obtained, therefore, with other enzymes of the same type. However, there is present also the possibility that salient properties of the

\* Published with the approval of the Director of the Experiment Station.



enzyme may be altered or destroyed in the process of separation and purification. In the present investigation, the inactivation temperature (10 minute heating period), and optimum reaction temperature of gluten proteinase and 0.04% papain in 0.1 *N* acetic acid were determined. In addition, the rate of activity of gluten proteinase at the optimum temperature was ascertained.

## EXPERIMENTAL

A commercial type hard red spring wheat flour containing 0.38% ash and 12.0% protein ( $N \times 5.7$ ) on 13.5% moisture basis was employed for preparing the gluten. The glutens were separated from the flour and dispersed in 0.1 *N* acetic acid by the method described by Harris and Johnson (1945). Briefly, this method involves dispersing 50 g. of crude wet gluten in 350 ml. of the acid in the Waring Blendor. Drying ovens, a dough fermentation cabinet and constant temperature baths were utilized for holding the dispersions at constant temperature levels when ascertaining the optimum reaction temperature of the two enzymes. The ovens and fermentation cabinet were susceptible of a temperature variation of  $\pm 1^\circ\text{C}$ . from the point at which they were set. The constant temperature baths, of course, had a more precise control. There is little doubt that the precision of temperature control was well within the accuracy limits of the determinations for proteolytic activity. The protein concentration of the dispersions was found by the Kjeldahl-Gunning method and, by subtracting the concentration of the dispersion after fractionation from the quantity of protein in the original, the quantity removed per 100 ml. of dispersion was obtained. Fractionation was accomplished by adding specified amounts of  $\text{K}_2\text{SO}_4$  and allowing the dispersions to stand approximately 18 hours at  $5^\circ\text{C}$ . to keep enzymatic activity at a minimum, before removal of the precipitated protein. The precipitated protein was removed by centrifuging for 20 minutes at 1850 r.p.m. in an International type SB centrifuge; the supernatant liquid was then decanted from the residual protein, and the protein content determined. Amino nitrogen was ascertained by the Sørensen formol titration method, as modified by Olcott, Sapirstein and Blish (1943) for use with acetic acid dispersions of gluten. Relative viscosity was found with the Hoeppler viscometer at  $25^\circ\text{C}$ .

Unheated dispersions reserved for the study of gluten proteinase activity were held at  $0^\circ\text{C}$ . to prevent proteolytic activity until used. Dispersions employed for the papain investigations were heated at  $90^\circ\text{C}$ . for 10 minutes with frequent agitation to inactivate the native proteinase (Harris and Johnson, 1945a). The papain, manufactured by Merck, was added in 1% suspension, without centrifuging, to the gluten dispersions. Care was exercised to thoroughly agitate the suspension before the removal of aliquots. Only freshly prepared suspensions were employed and these were thoroughly mixed with the gluten dispersion to insure maximum access of enzyme to the protein particles. Both  $\text{K}_2\text{SO}_4$  and papain were added in 0.1 *N* acetic acid to prevent any change in normality of the dispersion medium. The protein content of the dispersions was adjusted to compensate for dilution by the  $\text{K}_2\text{SO}_4$  and papain solutions.

To determine the inactivation temperature of the gluten proteinase and papain under the experimental conditions involved, the dispersions containing the active enzymes were heated at temperatures of 60°, 70°, 80°, 90° and 98°C., then allowed to stand 63 hours. Those employed for the gluten proteinase investigation were held at 50°C., while the papain-treated samples stood at 75°C. The dispersions used in the inquiry concerning the optimum temperature of activity of the two enzymes were also digested for 63 hours.

## RESULTS AND DISCUSSION

As in former studies on gluten dispersions by two of the authors (Harris and Johnson, 1945, 1945a), the data will be presented in the form of diagrams rather than in tables of precise values, because they are not quantitatively reproducible in different laboratories. This is particularly true of the amino nitrogen results, as it was found difficult in many instances to obtain satisfactory replications in the determination. There is little doubt, however, that repeated experimentation would reveal the general trends shown in the figures included in this report.

### *Inactivation Temperature*

Concentration of protein, amino nitrogen content, relative viscosity and the quantity of protein precipitated by 0.06 *N* K<sub>2</sub>SO<sub>4</sub> were determined on the dispersions containing active gluten proteinase (Fig. 1). Changes in these properties caused by proteinase activity while standing 63 hours at 50°C. can be estimated by comparisons between the heated and unheated dispersions. A temperature of 50°C. was used because it was presumed that optimum temperature of gluten proteinase activity would be rather high, resembling papain. Amino nitrogen content and fractionation data indicate that inactivation of the native flour proteinase was practically complete at 70°C. Relative viscosity did not prove as useful a measure of the influence of temperature of inactivation as amino nitrogen content and fractionation since changes in viscosity are caused by heating the dispersions (Olcott *et al.* 1943; Harris and Johnson, 1945a). The results secured from the dispersions in which the proteinase had been inactivated and then 0.04% papain added indicated that this enzyme had a higher inactivation temperature than the flour proteinase and required a temperature of approximately 90°C. to inhibit activity (Fig. 2). Viscosity measurements were not utilized for assessing the effects of

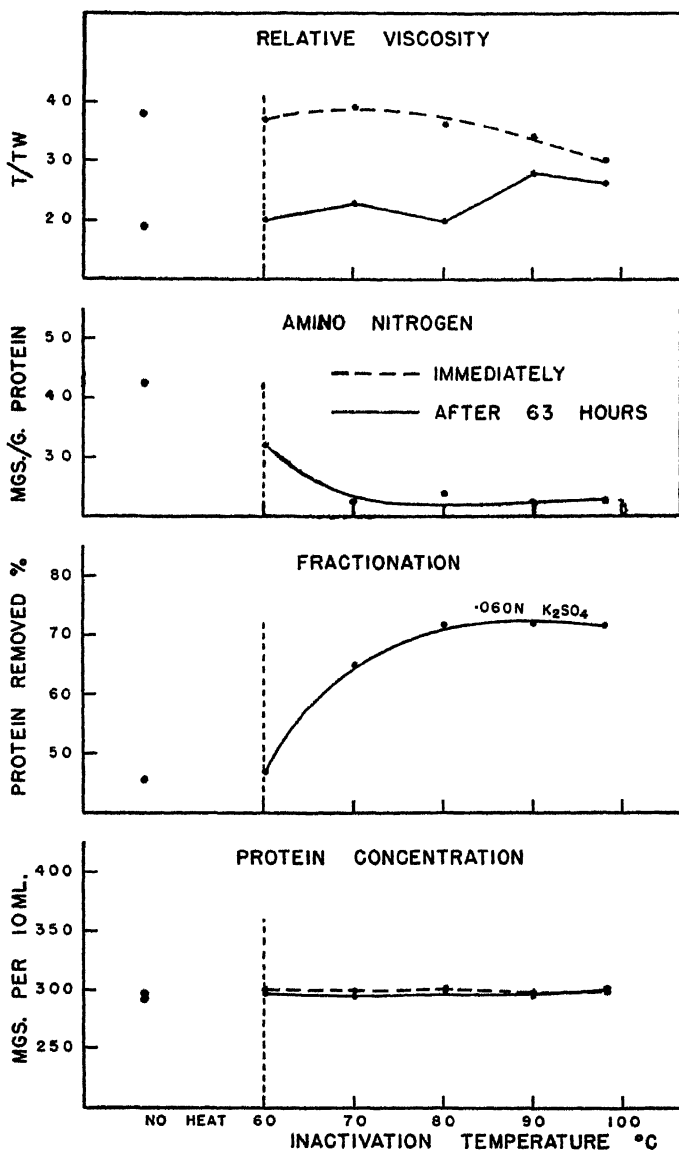


FIG. 1

Temperature of Inactivation After 10 Min. Heating Period of the Flour Proteinase, as Determined by Relative Viscosity, Amino Nitrogen Content and Protein Fractionation

heat on papain since the viscosity data secured from the flour proteinase determinations were not as useful as data obtained from the other determinations. It was decided to employ a temperature of 90°C. for 10 minutes in the following experiments.

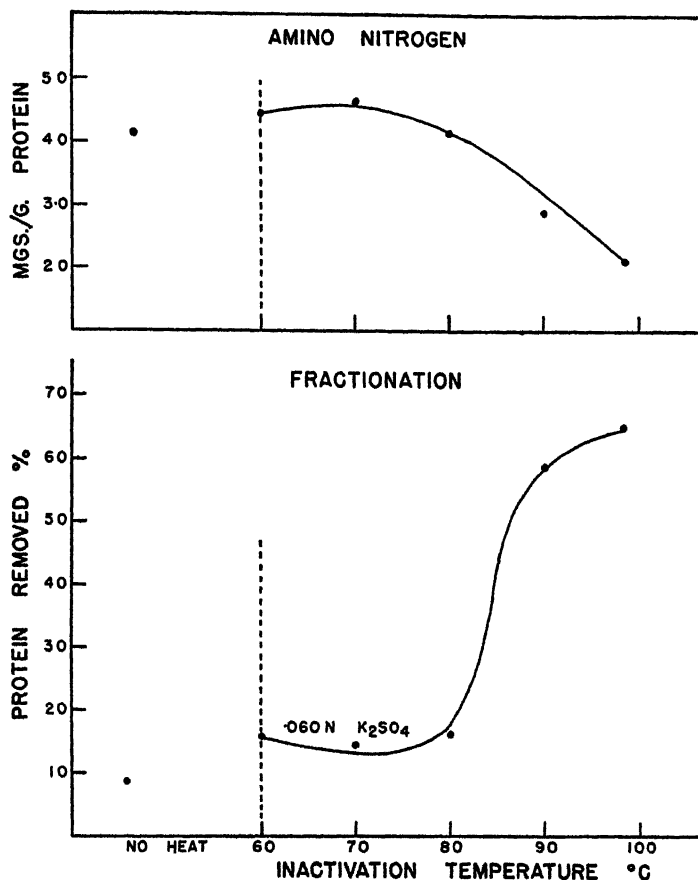


FIG. 2

Temperature of Inactivation After 10 Min. Heating Period of Papain, as Determined by Amino Nitrogen Content and Protein Fractionation

Olcott (private communication) has observed that inactivation at 95°C. was attained in 30 to 45 seconds by use of a flash pasteurizing apparatus. No doubt the destruction of enzyme activity is enhanced

in these dispersions by the rather exposed condition of the enzyme when adsorbed on the protein micelle as compared with the more usual situation where the enzyme is more protected from extraneous influences by association on larger aggregates of material. No effect on protein concentration was caused by heating.

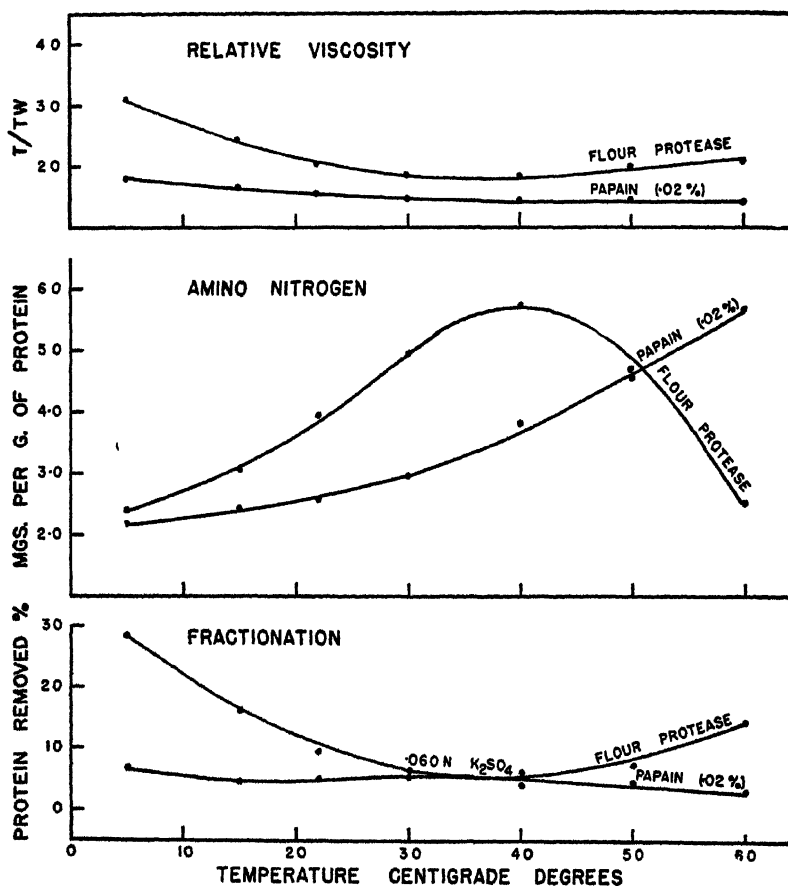


FIG. 3

Effect of Reaction Temperature on Activity of Flour Proteinase and Papain after 87 Hours as Registered by Changes in Relative Viscosity, Amino Nitrogen Content, and Protein Fractionation

*Optimum Temperature of Activity*

Determinations of enzyme activity were made at various temperature levels, using production of amino nitrogen, viscosity of the dispersions and protein precipitated by  $K_2SO_4$  as criteria of proteolytic activity. Similar determinations of papain activity on the heated gluten substrate at the same pH were also made (Figs. 3 and 4). Initial experiments employing the three criteria indicated a maximum proteinase activity in the neighborhood of 40°C., but the activity of papain was still increasing at 60°C. The fractionation trends, in

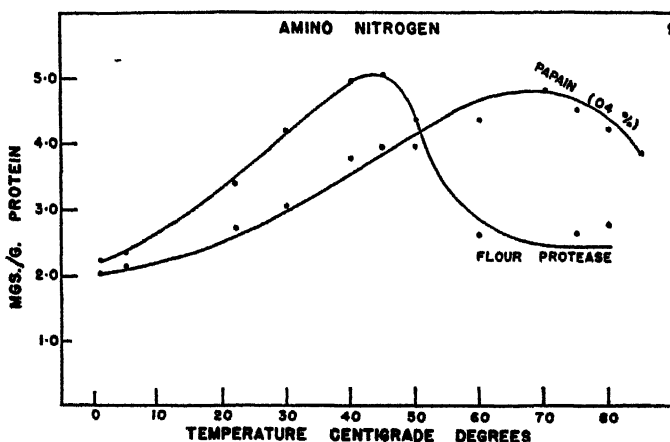


FIG. 4

Effect of Reaction Temperature on the Activity of Flour Proteinase and Papain, as Found After 63 Hours

particular, showed very small though significant changes among the temperatures employed. Observations over a larger temperature range were, therefore, obtained (Fig. 4) using amino nitrogen content only as a measure of enzyme activity since this property is more conveniently determined than protein fragmentation by fractionation technique. These more extensive data show that the optimum temperature for 0.4% papain is approximately 75°C., 30° higher than that for the native flour proteinase, and raises doubt that the two enzymes are as nearly alike in type as observations in the literature would lead one to anticipate. In support of this doubt, Olcott, Sapirstein and Blish (1943) pointed out that gluten proteinase was inactivated in 10%

sodium salicylate while the activity of commercial papain could be demonstrated in this substance. These investigators also found differences in solubility in dilute salt solutions and water between this gluten enzyme and the flour proteinase previously described by Hale (1939). There is little doubt that the pH (approximately 3.2) of the dispersions is well below the optimum for papain but close to the optimum for flour proteinase (Olcott *et al.*, 1943), but the effect of pH on the optimum should be very similar if the same type of enzyme is concerned in both reactions. It is possible, of course, that the gluten enzyme differs in respects other than solubility in water and saline solutions (Hale, 1939; Olcott, Sapirstein and Blish, 1943) from the flour proteinase isolated by Hale. Uncertainty also exists regarding the concentration of the gluten proteinase. There may be a possibility that the properties of the proteinase preparation used by Hale had been altered by isolation and drying, although it is scarcely likely that a change of 30°C. in temperature optimum would be due to these factors. Observations reported by other workers reveal 75°C. as the approximate optimum temperature for papain. The higher papain concentration, 0.04%, based on weight of wet crude gluten taken, gave results in closer agreement with those from the flour proteinase than the lower 0.02% papain treatment.

#### *Reaction Rate at Optimum Temperature*

The change in amino nitrogen content and proportion of protein removed by two concentrations of  $K_2SO_4$  with time, using unheated acetic acid dispersions at 40°C., are shown (Figs. 5 and 6) for a 36 hour period. Apparently the greatest enzymic activity takes place within the first 24 hours. Amino nitrogen production appeared to show the more significant changes, but the fractionation data, which should record the fragmentation of protein induced by the gluten proteinase, may be actually more important for the purpose of the investigation. Peptidases are the enzymes most concerned with the liberation of free amino groups, but both the native adsorbed flour proteinase and the papain must have carried the peptidase type of enzyme unless one admits the possibility of the proteases acting to produce the free amino groups. It was, of course, impossible to show the effect of enzyme concentration in relation to rate of activity since the proteinase remained adsorbed on the gluten.

The results secured in this investigation offer additional evidence that a proteinase exists in active form in acetic acid dispersions of gluten protein. This enzyme differs from papain in inactivation

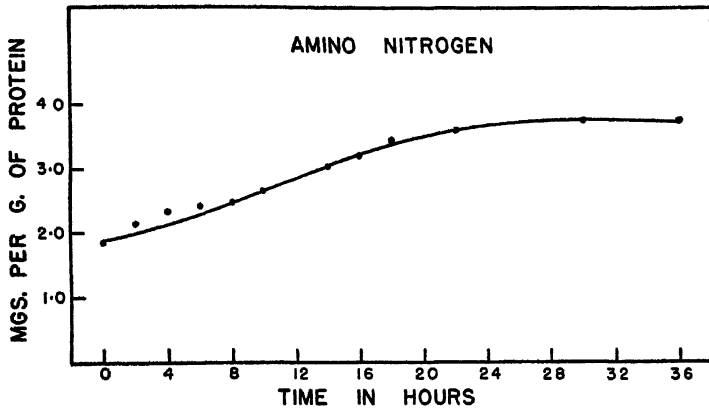


FIG. 5

Relation of Reaction Time of Flour Proteinase at 40°C. to Amino Nitrogen Content

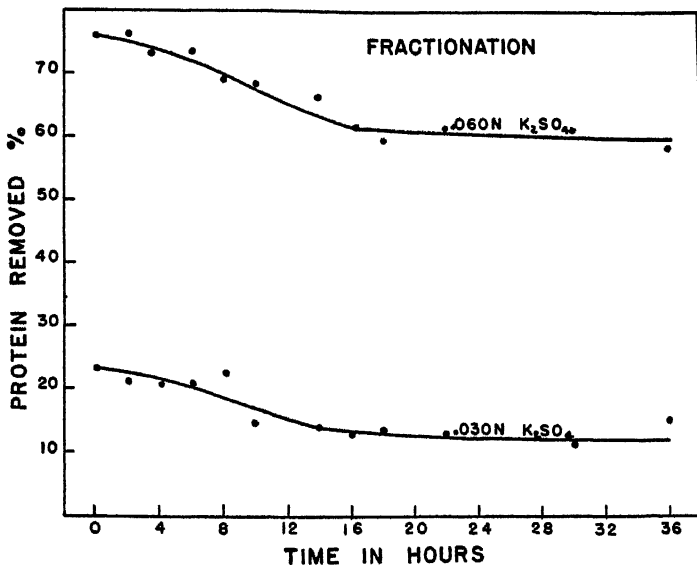


FIG. 6

Relation of Reaction Time of Flour Proteinase at 40°C. to Protein Fractionation



temperature and temperature of optimum activity. It is inactivated by heating for 10 minutes at 70°C., and is most active at pH 3.2 at a temperature of 40°C. At this temperature and pH, the activity ceases after 24 hours.

### SUMMARY

A comparison between native flour proteinase and papain in 0.1 *N* acetic acid (about pH 3.2) dispersions indicated distinct differences in temperature of inactivation over a ten minute period and temperature of optimum activity. For flour proteinase, these temperatures were approximately 70° and 40°C., respectively, while for papain the corresponding temperatures were 90° and 75°C. Proteolytic activity in the dispersions was measured by changes in relative viscosity, amino nitrogen content and proportion of protein removed by K<sub>2</sub>SO<sub>4</sub> additions. Amino nitrogen content appeared to show the largest differences of any of the determinations employed.

The activity of the gluten proteinase at the optimum temperature, 40°C., appeared to cease after 24 hours digestion. The activity of this enzyme is relatively weak and is probably different from the principal flour proteinase active in fermenting doughs.

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# A Note on the Amino Acids of Cataractous and Sclerosed Human Lenses

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## INTRODUCTION

The dry matter of the lens, in common with many other tissues of ectodermal origin, is composed primarily of protein. Salit (1) has shown that the nitrogen values, in terms of dry weight, are practically the same for all types of cataractous and sclerosed human lenses, even those considered "practically normal." On the basis of wet weight, however, the different types of cataractous and sclerosed lenses differ considerably in their nitrogen contents. Thus, the average N content for "practically normal" lenses was found to be 5.36%, decreasing to 4.83% during more pronounced sclerosis and finally to 4.43% during advanced sclerosis. When the data were arranged in the order of increasing severity of the cataracts, the amount of nitrogen decreased approximately 25% from "practically normal" lenses to mature cataracts. Salit (1) concluded that "there is, therefore, a considerable loss in protein in the senile pathological lens, as a result of cataract and pathological sclerosis, and the loss, on the whole, is proportionate to the degree of damage to the lens. Since the loss consists chiefly of the soluble proteins or crystallins, the insoluble albuminoids are left behind and impart to the nucleus, in which they predominate, an increased rigidity and hardness."

The following experiments were conducted to throw further light on this hypothesis.

## EXPERIMENTAL

Human cataractous lenses were collected in Iowa City and classified into three groups. Group I consisted of 40 lenses with incipient cataract and with 0, + and

++ sclerosis. Group II consisted of 42 lenses with incipient cataract and with +++ and ++++ sclerosis. Group III was made up of 37 lenses with mature cataracts, all highly sclerosed. The lenses of each group were placed in alcohol, crushed, repeatedly extracted with alcohol, followed by acetone and ether and dried in a vacuum desiccator over  $\text{H}_2\text{SO}_4$ . Yield: 2.84 g. Group I, 2.8 g. Group II, and 1.75 g. Group III.

The dried lens proteins were analyzed for nitrogen in duplicate by the micro Kjeldahl method, for tyrosine and tryptophane by Lugg's modification of the Millon procedure (*cf.* 2) (4 replicate experiments), for phenylalanine by a modification of the Kapeller-Adler method (*cf.* 2) (6 replicate experiments), for cystine in triplicate by the Folin method (*cf.* 2) and for methionine in 4 replicate experiments by the McCarthy-Sullivan procedure (*cf.* 2). Lysine, leucine, isoleucine and valine were estimated by the microbiological methods (3, 4, 5). In this case, ten testing levels were employed using two different microorganisms on separately prepared hydrolysates. Determinations for each amino acid in the three groups were carried out simultaneously and under the same conditions, in order to increase the comparative value of the results.<sup>1</sup> The analytical results, calculated from the nitrogen content of the "pure" lens protein (1), are given in Table I.

TABLE I  
*Amino Acids Yielded by Cataractous and Sclerosed Human Lenses*

Amino Acid	Group I <i>Per cent</i>	Group II <i>Per cent</i>	Group III <i>Per cent</i>
Tyrosine	$5.9 \pm 0.1$	$6.0 \pm 0.1$	$6.0 \pm 0.1$
Tryptophane	$3.3 \pm 0.1$	$3.4 \pm 0.1$	$3.4 \pm 0.1$
Phenylalanine	$8.5 \pm 0.2$	$8.8 \pm 0.4$	$8.6 \pm 0.2$
Cystine	$1.5 \pm 0.1$	$1.5 \pm 0.1$	$1.5 \pm 0.1$
Methionine	$3.5 \pm 0.3$	$3.8 \pm 0.2$	$3.6 \pm 0.3$
Lysine	$6.0 \pm 0.1^*$		
Leucine	$7.3, ^* 7.5^b$		
Isoleucine	$6.2, ^* 6.2^b$		
Valine	$5.3, ^* 5.3^b$		

\* 3 separate hydrolysates.

<sup>a</sup> *Streptococcus lactis*.

<sup>b</sup> *Lactobacillus arabinosus*.

## DISCUSSION

Although there do not appear to be any significant differences in the percentage composition of the amino acids in the three groups of lens proteins, the analytical findings are of interest. The relatively high content of the aromatic amino acids should be noted. In general, the amino acid pattern in lens proteins appears more nearly to resemble

<sup>1</sup> The majority of analyses were carried out by L. R. Knight.

blood fibrin, except for the lower lysine, than keratins and other proteins of ectodermal origin (*cf.* 2).

The amino acid analyses do not support the hypothesis that the loss in protein in the senile pathological lens "consists chiefly of the soluble proteins," unless it is also assumed that the amino acid pattern of the crystallins and albuminoids is approximately the same. An alternative explanation, to account for the relative decrease in crystallins, is to assume that the amino acid pattern of crystallins and albuminoids are the same or very similar, and that the differences in physical properties are due to denaturation (*cf.* 6).

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# Electrophoretic Analysis of Bovine Plasma and Serum

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## INTRODUCTION

Electrophoretic studies of plasma and serum proteins of man and of various animals have shown rather marked variations in the distribution of these blood proteins among different species (1-4). Longworth (5) reported the electrophoresis of human and horse plasma in a variety of buffer solvents and found sodium diethylbarbiturate (veronal) most efficient for the analysis of human plasma, while a phosphate buffer gave a more satisfactory pattern for horse plasma. Moore (4), using veronal and phosphate buffers, found that the serum protein patterns of several species are dependent upon the buffer solvent.

It was the purpose of this investigation to study the resolution and relationship of bovine plasma and serum components in phosphate and veronal buffers.

## EXPERIMENTAL

The electrophoretic technic and methods of determining mobilities and percentage composition of the plasma and serum samples were those described in a previous report (6).

Phosphate, veronal-NaCl, veronal and veronal-citrate buffers were compared for efficiency in resolving the protein components of bovine plasma. The compositions of the buffers have been described (6). The physical constants for each of these solvents are given in Table I. Duration of the electrolysis in each solution was determined by the migration of the fastest component, albumin, to approximately 6 cm. from the initial boundary.

Dried pooled bovine plasma was used for this study. Experiments in this laboratory have shown that the electrophoretic patterns of bovine plasma, analyzed before and after drying, are identical. Determinations made over a period of several months

\* Now in United States Navy.

on dried plasma samples, kept at 27°F., have shown no evidence of changes in the boundary patterns.

Whole blood from 200 animals was collected at the slaughter house with the addition of 1 volume of 4% sodium citrate solution to 10 volumes of blood. After separation from red cells by continuous centrifugation, the plasma was dried in the frozen state.

TABLE I  
*Mobilities of Compounds of Whole Plasma*

Buffer	pH	1/2	K ×10 <sup>3</sup>	V/cm.	Time in minutes	Albu- min	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2$	$\phi$	$\gamma$
Phosphate	7.7	0.2	6.60	4.55	240	5.70		4.45		3.48	2.62	1.83
Phosphate	7.7	0.2	6.60	4.55	240	5.77		4.49		3.51	2.66	1.85
Veronal-NaCl	8.5	0.2	8.34	3.60	345	5.27	4.44	3.74		2.88	2.30	1.54
Veronal-NaCl	8.5	0.2	8.34	3.60	345	5.28	4.49	3.70		2.89	2.31	1.50
Veronal	8.6	0.1	3.05	6.55	150	6.34	5.39	4.67	3.81	3.09	2.44	1.73
Veronal	8.6	0.1	3.05	6.55	150	6.38	5.40	4.67	3.84	3.15	2.45	1.71
Veronal-citrate	8.5	0.1	2.86	7.00	120	6.54	5.45	4.62	3.84	3.24	2.51	1.55
Veronal-citrate	8.5	0.1	2.86	7.00	120	6.52	5.38	4.62	3.81	3.24	2.51	1.52

For the electrophoretic comparison of bovine plasma and serum in the phosphate buffer and in veronal buffer, ionic strength 0.1, the blood of one animal was used. It was collected in two containers at the time of killing. One sample was treated with sodium citrate as described above. The other was stirred continuously for 5-10 minutes until the fibrin had collected on the glass stirring rod and could be removed. Both samples were then centrifuged.

The dried and liquid plasma and serum samples were prepared for electrolysis in the same manner. Solutions of 2.5% protein concentration were made in the appropriate buffer and dialyzed in visking casings at 40°F. against 40 volumes of buffer with daily changes of buffer for 3 days or until there was no change in the conductivity of the dialysate. After filtering, the protein solutions were adjusted to 2% concentration by measuring the index of refraction.

## RESULTS

A typical boundary pattern of the dried plasma in each buffer is given in Fig. 1.

The large fibrinogen,  $\phi$ , peak and the poor resolution of the  $\phi$  and  $\gamma$ -globulin are characteristic of these patterns. The  $\beta$ -globulin is clearly resolved from the fibrinogen only in the phosphate buffer. The  $\alpha$ -globulin boundary in this solvent is a single, asymmetric curve and the descending pattern shows a marked spreading of the albumin peak.

In veronal-NaCl the  $\beta$ -globulin appears as a shoulder on the steep fibrinogen peak. A faster  $\alpha_1$ -globulin is separated from the larger  $\alpha_2$ -globulin. The albumin curve of the descending pattern is only slightly spread.

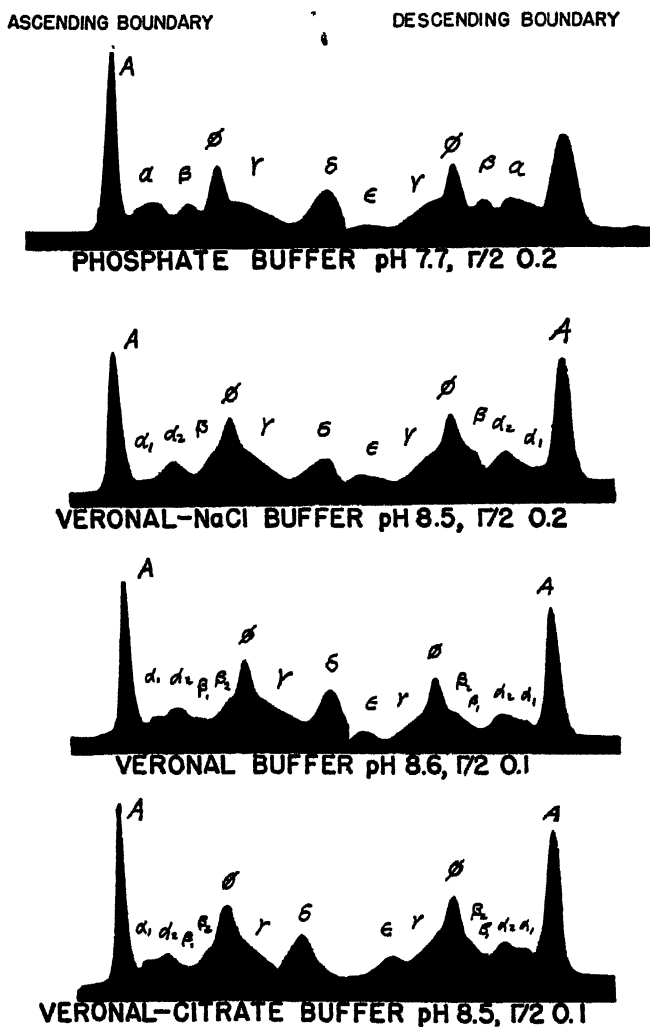


FIG. 1  
Whole Plasma



The veronal and veronal-citrate patterns are very similar. The  $\beta$ -globulin appears as two curves. The slower,  $\beta_2$ , component is poorly separated from fibrinogen. Globulins  $\alpha_1$  and  $\alpha_2$  are clearly defined, and there is little diffusion of the descending albumin boundary.

In every buffer the  $\gamma$ -globulin is well separated from the  $\delta$ ,  $\epsilon$  anomalies, and the ascending and descending boundary patterns are good mirror images.

Mobilities and percentage distribution of the plasma proteins are reported for duplicate runs in each buffer in Tables I and II respectively.

The apparent concentration of the  $\gamma$ -globulin, determined in bovine plasma by electrophoresis, is relatively low and shows little variation in the different buffers. There is less than 2% variation in the percentage concentration of fibrinogen found in the various buffers. By this method of analysis, fibrinogen equals about one-fifth of the total plasma protein. The experiments in veronal-NaCl indicate a smaller ratio of  $\alpha_1$ - and  $\alpha_2$ -globulin than the veronal and veronal-citrate analyses show.

The electrophoretic determinations in phosphate buffer reveal the highest albumin concentration and lowest  $\alpha$ -globulin content, while the veronal-NaCl analyses gave the lowest albumin and highest total  $\alpha$ -globulin concentrations.

The albumin to globulin ratio, A/G, for each experiment is listed in the last column of Table II.

TABLE II

*Percentage Distribution of Total Plasma Protein in Various Buffers*

Buffer	Albumin	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2$	$\phi$	$\gamma$	A/G
Phosphate	38.3		15.6		10.6	20.1	15.4	.62
Phosphate	38.8		15.4		10.1	20.4	15.3	.63
Veronal-NaCl	34.1	4.6	12.8		11.4	21.2	15.9	.52
Veronal-NaCl	34.3	4.2	12.4		12.1	21.6	15.4	.52
Veronal	36.2	5.5	10.9	3.0	9.9	19.9	14.6	.57
Veronal	35.4	5.6	10.4	3.9	9.5	19.8	15.4	.55
Veronal-citrate	36.7	6.0	9.8	3.3	7.8	20.9	15.5	.58
Veronal-citrate	35.7	5.9	10.0	3.8	7.8	20.9	15.9	.56

The plasma and serum of one animal were compared by electrophoresis in phosphate and veronal buffers. Typical boundary patterns

in phosphate are given in Fig. 2, and in veronal, Fig. 3. It is seen that in each buffer the same number of peaks occur in the serum pattern as in the plasma pattern. Table III shows that the mobilities of the corresponding peaks are the same.

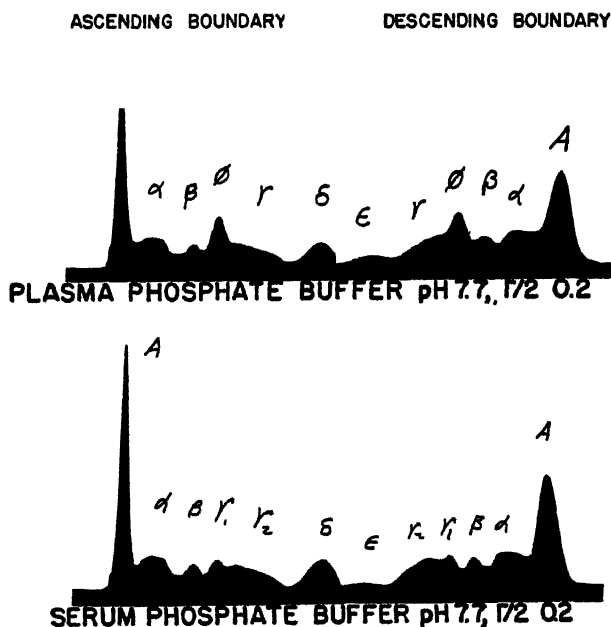


FIG. 2  
Plasma-Serum (Phosphate Buffer)

TABLE III  
*Mobilities (Plasma-Serum)*

Buffer	Sample	Albumin	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2$	$\phi$	$\gamma_1$	$\gamma_2$
Phosphate	Plasma	5.68		4.47		3.46	2.68		1.79
Phosphate	Plasma	5.70		4.54		3.47	2.68		1.82
Phosphate	Serum	5.74		4.56		3.42		2.69	1.83
Phosphate	Serum	5.70		4.50		3.40		2.69	1.81
Veronal	Plasma	6.20	5.30	4.63	3.61	2.92	2.34		1.58
Veronal	Plasma	6.23	5.37	4.65	3.72	2.98	2.42		1.63
Veronal	Serum	6.19	5.27	4.62	3.67	2.86		2.38	1.72
Veronal	Serum	6.17	5.22	4.54	3.62	2.84		2.34	1.61

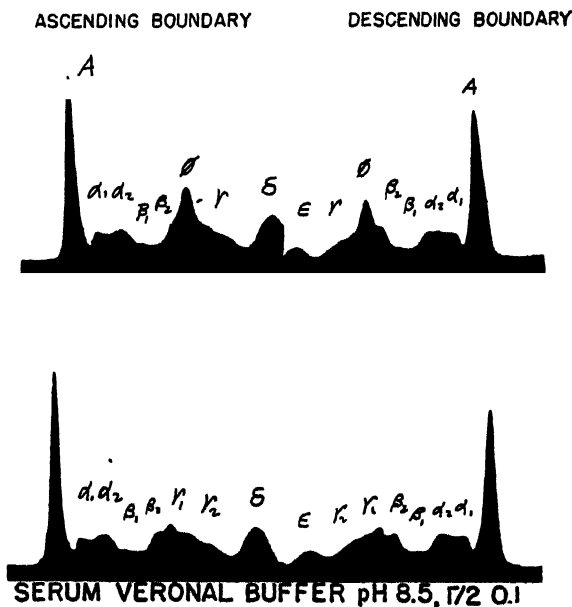


FIG. 3

Plasma-Serum (Veronal Buffer)

The percentage distribution of the plasma and serum proteins calculated from these experiments are reported in Table IV.

### DISCUSSION

The spreading of the albumin boundary in the descending electrophoretic pattern seems to be typical of the phosphate buffer (5, 6) and the lower  $\alpha$ -globulin concentration in phosphate indicates that the larger A/G ratio found in this buffer may be due to the masking of part of the  $\alpha$ -globulin by albumin.

Variation of the fibrinogen and  $\beta$ -globulin concentrations in the veronal-containing buffers may be partially explained by the poor resolution of these components.

The electrophoretic comparison of the plasma and serum of one animal shows that actually another component, which we believe is part of the  $\gamma$ -globulin, is migrating with a mobility very close to that of fibrinogen. The peak labelled  $\phi$  in the whole plasma patterns then includes this  $\gamma_1$ -globulin, and the percentage of fibrinogen in the total

plasma protein cannot be determined simply by measuring the area under this  $\phi$  peak.

It is possible, however, to calculate the percentage of fibrinogen in the total plasma protein from the percentage of albumin in the total protein of plasma and of serum respectively, as determined by electrophoretic analysis, if it is assumed that the only difference between the plasma and serum, prepared from the blood of one animal by the methods described above, is the absence of fibrinogen from serum.

If, then, only the  $\phi$  is removed from plasma to make serum, the ratio of the amount of albumin to the amount of  $\alpha$ -globulin, or  $\beta$ -globulin, or  $\gamma$ -globulin is not changed. Where A,  $\alpha$ ,  $\beta$ , and  $\gamma$  are the concentrations of albumin and  $\alpha$ ,  $\beta$ , and  $\gamma$ -globulin respectively, then

$$\frac{A(\text{plasma})}{(A + \alpha + \beta + \gamma)_{\text{plasma}}} = \frac{A(\text{serum})}{(A + \alpha + \beta + \gamma)_{\text{serum}}} \quad (1)$$

By multiplying the right half of equation (1) by 100, we obtain  $A_s$ , the percentage of albumin in total serum protein, and:

$$\frac{A(\text{plasma})}{(A + \alpha + \beta + \gamma)_{\text{plasma}}} \times 100 = A_s \quad (2)$$

The concentrations A,  $\alpha$ ,  $\beta$ , and  $\gamma$  in plasma can be expressed as percentages of total plasma protein. So, if we let the total protein of plasma equal 100%,  $\phi_p$  equal the percentage of fibrinogen, and  $A_p$  equal the percentage of albumin, equation (2) becomes:

$$\frac{A_p}{100 - \phi_p} \times 100 = A_s \quad (3)$$

Solving for  $\phi_p$  we obtain:

$$\phi_p = 1 - \frac{A_p}{A_s} \times 100 \quad (4)$$

The albumin concentrations are used in these calculations because albumin is the component in plasma and serum present in the greatest concentration and is most clearly resolved in the electrophoretic patterns. However,  $\alpha$ ,  $\beta$  or  $\gamma$  could be substituted for A in the numerator of both sides of equation (1), if adequate resolution of these components was possible in an electrophoretic pattern.

Two *per cent* solutions were used in the electrophoretic analysis of the plasma and serum to standardize the conditions of the experiments, for there is evidence (7, 8) that, in this method of analysis, the resolution of the different components varies with the total protein concentration in the solution analyzed. But since the components are expressed as the percentage of the total protein, these values are, in the ideal case, numerically the same whether determined in a total protein solution of 1%, 2%, 3%, *etc.*

According to Table IV, the determinations in phosphate buffer give an average concentration of albumin in plasma protein, ( $A_p$ ), of 38.75%, and in serum protein of 42.8%. Substituting these values in equation (4), we find that  $\phi_p$  is 9.46% of the total protein in plasma.

TABLE IV  
*Percentage Distribution of Proteins (Plasma-Serum)*

Buffer	Sample	Albumin	$\alpha_1$	$\alpha_2$	$\beta_1$	$\beta_2$	$\phi$	$\gamma_1$	$\gamma_2$
Phosphate	Plasma	38.4		19.0		8.7	16.7		17.2
Phosphate	Plasma	39.1		19.1		8.9	16.3		16.6
Phosphate	Serum	42.8		19.5		10.6		8.9	18.2
Phosphate	Serum	42.8		19.5		9.8		8.8	19.1
Veronal	Plasma	35.9	6.8	12.7	4.1	8.1	16.1		16.4
Veronal	Plasma	35.4	6.8	13.0	4.3	8.3	16.3		16.0
Veronal	Serum	38.0	7.5	12.9	7.3	9.6		6.5	18.2
Veronal	Serum	38.0	8.4	11.4	7.0	9.0		7.6	18.6

A similar calculation using the concentration of albumin found by analysis in veronal buffer (Table IV) gives the fibrinogen as 6.2% of the total plasma protein.

As a compromise, it can be said that by this method of determination the fibrinogen in plasma constitutes between 6.2% and 9.46% of the total protein, depending upon the buffer used in the analysis.

The authors are indebted to Professor F. C. Koch and Dr. J. B. Lesh for their encouragement and helpful suggestions during this work.

### SUMMARY

Bovine plasma has been studied by electrophoretic analyses in phosphate, veronal-NaCl, veronal, and veronal-citrate buffers.

A satisfactory resolution of  $\gamma$ -globulin and fibrinogen was not achieved in any of these solvents. In each instance the fibrinogen content was exceedingly high at the expense of the  $\gamma$ -globulin concentration. The phosphate buffer was superior for the separation of the  $\beta$ -globulin from  $\phi$ . Veronal and veronal-citrate, ionic strength 0.1, patterns showed a faster  $\beta_1$ -globulin, but the  $\beta_2$ -globulin appeared as a shoulder on the  $\phi$  peak. The veronal-NaCl buffer was found most efficient for the separation of total  $\alpha$ -globulin from albumin. The best resolution of  $\alpha_1$ - and  $\alpha_2$ -globulin was found in veronal and veronal-citrate buffers.

The plasma and serum from one cow were analyzed by electrophoresis in phosphate and veronal buffers. This study revealed the migration of part of the  $\gamma$ -globulin with the fibrinogen. The fibrinogen content of bovine plasma was calculated to be from 6.2% to 9.5% of the total plasma protein.

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# ***l*-Galacturonic Acid from *d*-Galactose**

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## **INTRODUCTION**

From a mechanism study (unpublished) of the oxidation of *d*-galactose by nitric acid, we concluded that the production of mucic acid followed the pathway *d*-galactose  $\rightarrow$  *d*-galactonic acid  $\rightarrow$  *l*-galacturonic acid  $\rightarrow$  mucic acid. The separation of the *l*-galacturonic acid from the galactonic acid and from a further oxidation product of mucic acid was impossible until Isbell and Frush (1) discovered that the sodium strontium salt of *d*-galacturonic acid has a limited solubility in water. This led us to try the same property for the isolation of *l*-galacturonic acid. Direct precipitation from the reaction mixture proved successful for the isolation of the sodium strontium salt of the *l*-compound. From the mixed sodium strontium salt the free acid was then easily prepared. Thus, *l*-galacturonic acid becomes a readily available uronic acid.

The only synthesis of *l*-galacturonic acid heretofore reported was given by Niemann and Link (2). Their synthesis started with the rare sugar, *l*-galactose. The preparation by way of the diacetone *l*-galactose was a relatively laborious process.

At one time Kiliani (3) had reported the isolation of *l*-galacturonic acid from the nitric acid oxidation of *d*-galactonic acid. Later, however, he (4) retracted this statement and admitted that he had mistaken the semicarbazide of galactonic acid for the desired product.

## **EXPERIMENTAL**

### ***Preparation of Sodium Strontium l-galacturonate***

One hundred grams of *d*-galactose were placed in a 2 liter Erlenmeyer flask with 100 ml. of concentrated nitric acid (sp. g. 1.42) and 100 ml. of water. After shaking to effect maximum solution, the flask was fitted with a stopper carrying a bent glass tube and placed in a water bath at room temperature for 24 hours. The glass tube was arranged as a water trap to prevent oxygen from gaining access to the reaction.



At the end of 24 hours the solution was filtered through a sintered glass funnel. One-third of the solution was neutralized with sodium bicarbonate and recombined with the remainder which was then completely neutralized to Congo red with strontium carbonate. Any excess strontium carbonate or any precipitated strontium salts were removed by filtration. After standing for several days precipitation of the mixed sodium strontium salt was complete. The dried salt, as the hexahydrate, after filtering and washing with ice water, weighed 17 g. Recrystallization was effected by dissolving the salt in 300 ml. of hot water, decolorizing with charcoal and allowing the solution to stand overnight. Yield, 8.5 g. of pure compound. A further quantity, 5.5 g., was obtained from the filtrate of the recrystallization by adding 300 ml. of methanol.

The rotation  $[\alpha]_D^{25}$  of the hexahydrate was  $-36.3^\circ$  (C, 1 in water). Isbell and Frush (1) reported  $+30.2^\circ$  for the *d*-isomer.

Anal.: Calcd. for  $\text{NaSr}(\text{C}_6\text{H}_9\text{O}_7)_2 \cdot 6\text{H}_2\text{O}$ : Sr, 10.98%; Found: 11.10.

#### *l*-Galacturonic Acid from the Sodium Strontium Salt

Seven g. of sodium strontium *l*-galacturonate were treated with 52.2 ml. (slightly less than theory) of 0.5 *N*  $\text{H}_2\text{SO}_4$ . After thorough stirring, 700 ml. of absolute alcohol were added. The solution was allowed to stand for 1 day and then filtered to remove sodium sulfate, strontium sulfate and excess starting material. On concentrating to dryness the *l*-galacturonic acid crystallized immediately. Yield 5.1 g. (98% theory). In most instances the product was free of inorganic matter and titrated correctly for the pure monohydrate. For synthetic work purification was unnecessary.

Purification may be effected by dissolving the acid in a small quantity of water and adding enough absolute alcohol to bring the concentration to 90% alcohol.

Anal.: Calcd. for  $\text{C}_6\text{H}_{10}\text{O}_7 \cdot \text{H}_2\text{O}$ ; N. E. 47.20 ml.

0.1 *N* alkali; C, 33.96, H, 5.66;

Found N. E. 47.15 ml.

0.1 *N* alkali; C, 33.80, H, 5.50.

The rotation  $[\alpha]_D^{25}$  was  $-51.9^\circ$  (c, 1 in water). Niemann and Link (2) reported  $-56^\circ$ .

#### DISCUSSION

The uronic acids are of considerable importance in nature. *d*-Gluconic acid is a constituent of plant gums, mucoids and immunopolysaccharides, *d*-mannuronic is found in a polysaccharide from sea algae, and *d*-galacturonic acid is the main sugar of the pectin molecule. Although a number of aldo- and keto-uronic acids have been synthesized, only one alduronic acid, *d*-galacturonic acid, has been obtained by a convenient preparation.

By isolating the *l*-galacturonic acid which arises in the mucic acid preparation from *d*-galactose this uronic acid is now readily obtained in pure form. The physical properties of *l*-galacturonic acid and of the sodium strontium salt coincide with those of the *d*-compounds, except that the optical rotations are of opposite sign.

## SUMMARY

The mixed sodium strontium salt of *l*-galacturonic acid was crystallized directly from a nitric acid oxidation mixture of *d*-galactose.

Upon decomposition with sulfuric acid, the sodium strontium salt yielded *l*-galacturonic acid in relatively pure form and in good yields.

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# The Effects of Baking and Toasting on the Nutritional Value of Proteins

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## INTRODUCTION

The importance of supplying a high-protein diet to presurgical and postsurgical patients (1, 2, 3) indicated the need of furnishing extra protein of high biological value in a palatable form to sick and convalescing individuals. The proteins chosen for this study are available in large quantities and are mutually supplementary. Thus, the maximal nutritive value would be achieved with the minimal quantity of protein ingested.

The plan was to so modify white flour that the resulting food would contain approximately 25% of its calories as protein with a distribution of essential amino acids approximating that of whole egg. The proteins of egg are among the highest in nutritive value.

## EXPERIMENTAL

After numerous trials, the following mixture was arrived at as most nearly fulfilling the objectives.

Cake Mix			
Ingredient	Amount	Calories	
Flour	510 g.	1810	
Sugar	197 g.	788	
Egg White	115 g.	460	
Lactalbumin	76 g.	308	
Hydrogenated Vegetable Oil	52 g.	468	
Dried Yeast	30 g.	100	
Molasses	11 g.	31	
Salt	9 g.	—	

\* The Assay experiments were aided by grants from The John Henry and Mary R. Markle Foundation, The Douglas Smith Foundation for Medical Research of the University of Chicago, and The National Livestock and Meat Board.

The mixture contains approximately 4000 calories per kg., of which carbohydrate furnishes 64%, protein 25% and fat 11%. The distribution of essential amino acids found in the cake mix proteins, compared to those found in spray-dried whole egg protein, is given in Table I. Arginine, histidine and lysine were estimated by the micro-Kossel method (*cf.* 4); tryptophane by the Millon-Lugg procedure (*cf.* 4); cystine and methionine by the Folin and McCarthy-Sullivan methods (*cf.* 4); threonine according to Nicolet (*cf.* 4); and leucine, isoleucine and valine by the microbiological technique (*cf.* 4).

TABLE I  
*A Comparison Between the Amino Acid Composition  
of Cake Mix and Egg Proteins*

	In 1 kg. of Cake Mix	In 100 g. of Cake Mix Protein	In 100 g. of Egg Protein
	g.	g.	g.
Arginine	11.3	4.9	6.4
Histidine	5.3	2.3	2.1
Lysine	15.0	6.5*	7.2
Tryptophane	3.9	1.7	1.5
Phenylalanine	12.5	5.4	6.3
Cystine	6.0	2.6	2.4
Methionine	7.6	3.3	4.1
Threonine	9.5	4.2	4.3
Leucine	20.8	9.0	9.2
Isoleucine	15.0	6.5	8.0
Valine	14.6	6.3	7.3

\* Microbiological determination of lysine on original mix indicated 6.3% of lysine; on toasted cake mix C 5.8% of lysine.

To every 3000 g. of cake mix, 3 cakes of compressed yeast, 2 teaspoons of grated lemon rind and 2 teaspoons of nutmeg were added, together with sufficient water (*ca.* 920 ml.) to make a proper batter. The dough was allowed to rise in a warm room (32-35°C.) over night, rolled to approximately 1 inch in thickness and again allowed to rise to a height of about 2 inches. Portions of the dough were then baked in greased cooking sheets in an oven at approximately 200°C. for 15 to 20 minutes. The resulting cakes were sliced in 1-inch slices and a portion was dried on the radiator for one day. The remaining slices were toasted in a low oven (*ca.* 130°C.) until they had the appearance of commercial rusk (Zwieback). This usually required 40-60 minutes. Both the air-dried cake and the toasted cake have excellent keeping properties and are very palatable. From the data in Table I, the proteins in the cake mix should have a high biological value.

The samples to be assayed for protein nutritive value were mixed into basal rations at comparable protein levels. The rations were then fed to protein-deficient adult white rats for 14 days to determine their potentiality for engendering weight-recovery (5). The basal ration con-

tained the following ingredients per 100 g.: corn starch, 71 g.; ruffex (Fisher), 5 g.; corn oil, 4 g.; salt mixture, 4 g.; liver concentrate, 1 g.; water, 15 ml.; calcium pantothenate, 1275  $\gamma$ ; pyridoxine HCl, 600  $\gamma$ ; riboflavin, 830  $\gamma$ ; thiamin, 540  $\gamma$ ; niacin, 1340  $\gamma$ ; oleum percomorphum (Vitamin A, 400 U.S.P. units, Vitamin D, 29 U.S.P. units); choline chloride, 180 mg. To this mixture the food materials to be tested were added so as to make the final protein concentrations in each ration approximately 9% ( $N \times 6.25$ ). At the same time the necessary readjustments were made for roughage, water and calories to make each ration isocaloric. The completed rations were fed to the rats in individual cages in 15 g. portions/rat/day and the food-consumption recorded. At the end of the feeding period, the "protein efficiency" (PE) was calculated from the g. of body weight gained/g. of protein eaten. As standards of reference, lactalbumin 15-42 and 1:1 mixture of lactalbumin and casein (vitamin-test, Smaco) were fed under comparable conditions in the same basal ration and at a 9% protein level (Table II). Weight changes on the basal diet alone are also given.

TABLE II

*The Effect upon Weight Recovery of Protein-Starved Rats of Raw, Baked, Toasted and Lysine-Fortified Cake Mix Samples, Compared with the Basal Ration and Other Protein Supplements, Fed over Fourteen Days*

No. of Rats	Diet	Protein Intake		Weight gain or loss		Protein Efficiency
		Range	Average	Range	Average	
6	Basal	1.0 to 1.2	1.1	-14 to -20	-22	
5	Lactalbumin	13.6 to 14.6	14.0	38 to 45	41	2.9
5	Lact.-Casein (1:1)	18.8 to 20.1	19.3	66 to 77	72	3.6
5	Lact.-Casein (1:1)	21.5 to 22.5	21.9	68 to 76	72	3.3
4	Raw cake mix B	19.1 to 19.4	19.2	62 to 70	67	3.5
8	Raw cake mix C	18.4 to 20.5	19.6	58 to 73	62	3.3
4	Baked cake mix B	18.8 to 19.6	19.2	45 to 46	46	2.4
4	Baked cake mix C	11.5 to 15.9	13.5	2 to 23	9	0.6
5	Toasted cake mix A	13.8 to 17.0	15.2	-2 to 15	3	0.2
4	Toasted cake mix B	15.5 to 17.2	16.4	4 to 21	13	0.8
4	Toasted cake mix C	12.4 to 14.6	13.5	-2 to 29	10	0.7
10	Toasted cake mix D	11.3 to 15.8	12.7	-8 to 5	0	0
4	Baked cake mix C plus lysine (0.49%)	17.5 to 20.8	19.3	41 to 60	52	2.7
4	Toasted cake mix C plus lysine (0.63%)	18.9 to 21.5	20.3	55 to 70	64	3.2

The effect of feeding the toasted "cake-mix" (A) for 14 days is shown in Table II. Although the animals, on the average, ate a little more protein than did those on the lactalbumin diet (Table II), nevertheless their growth-performance was poor. Four of the animals remained practically stationary in weight while one gained slightly. In other words, the nutritional value of the toasted sample was markedly lower than lactalbumin, even though the consumption of the latter protein was slightly less.

To ascertain whether or not the heating had caused this poor result, a second "cake-mix" was prepared and divided into three portions for further testing, as follows:

B. Original cake-mix, unheated

Original cake-mix, baked and air dried

Air-dried sample toasted at approximately 120°C. for 1 hour.

These three samples, after proximate analysis, were mixed into the basal ration and fed as before to three more groups of protein-deficient rats. The results are shown in Table II, diets B, where it is seen that the protein-quality of the original unheated mix was high. In fact, the PE of the mix was better than that of lactalbumin alone and equal to the lactalbumin-casein mixture (*cf.* Table II). The proteins in the baked cake were significantly inferior while the PE of the toasted sample were markedly lower than the original mix. It is evident, therefore, that processing caused deterioration of the proteins in the "cake-mix," especially in the toasted sample (Toasted Cake Mix B).

In view of the probability that the toasting temperature had affected some amino acid in the protein, most probably lysine, a third experiment was performed. Three additional samples of "cake-mix" were prepared as follows:

C. Original mix, unheated

Original mix, baked and air dried at 60°C. overnight in a commercial dehydrator

Baked and air dried mix supplemented with lysine in amount equal to 0.36% of the final ration

Original mix, baked and toasted at 100°-125°C. for 60-75 minutes

Toasted material supplemented with lysine in an amount equivalent to 0.63% of the final ration.

After proximate analysis, the samples were mixed into the basal ration as usual. These five rations were then fed to five groups of protein-deficient rats for 14 days. The results are shown in Table II.

It is obvious that the animals eating the two processed samples did poorly, despite the divergent responses of two rats. These two animals ate about 2 g. more of protein during the second week than did the others. Because of this, two further samples of rusk were prepared six months later. The rusk was fed to two groups of depleted animals and the combined results are given in Table II, diet D.

Of greater significance is the fact that the animals eating the lysine-supplemented samples showed a marked improvement in weight recovery and food consumption. In fact, those getting the larger supplement of lysine in the toasted sample practically equalled the performance of the animals eating the original "cake-mix." The amount of lysine added was decided upon as follows: The ration probably contains at least 0.50 g. of lysine/100 g. because the addition of the unheated "cake-mix" to the basal diet, at a 9% protein level, gives a minimal lysine value of 0.49%. Assuming that most of the lysine in the toasted sample had been inactivated, and allowing for the fact that the lysine analyses by the Kossel method may have been low, 0.63% lysine (0.86 g. of lysine-HCl) was added to give an equivalent of 7% of the protein in the ration. On the assumption that there was less deterioration in the baked sample, only enough lysine was added to bring the concentration to 4% of the protein. The experimental results, Table II, indicate that insufficient lysine was added in this experiment, *i.e.*, baking plus drying at 60°C. in a dehydrator (Baked Cake Mix C) had resulted in a greater deterioration than drying on the radiator (Table II, Baked Cake Mix D).

## DISCUSSION

The injurious effects of dry heat, especially of toasting, on the nutritive value of many proteins has been repeatedly demonstrated by Morgan (6, 7), Murlin (8), Mitchell (9), Stewart (10) and others. Although heat treatment may, in some cases, decrease digestibility, as evidenced by increased fecal nitrogen, the major cause of heat injury appears to be the result of an impaired assimilation of one or more essential amino acids. Thus, Greaves, Morgan and Loveen (7) found that heated casein could be almost restored to its original



biological value by the addition of lysine. Similar results of the sensitivity of lysine to dry heat in lysine-rich human globin were obtained by Devlin and Zittle (11). Dry heating of purified proteins does not, however, result in the chemical destruction of lysine, for Block, Jones and Gersdorff (12) have shown that as much lysine could be isolated after acid hydrolysis of heated casein as from unheated, while Seegers and Mattill (13) found that acid hydrolysis of heat-impaired proteins restored their nutritive value when tryptophane, alone, was added. Partial destruction of lysine may, however, occur during toasting of foodstuffs in the presence of large quantities of carbohydrates and fats. A possible explanation (*cf.* 14) of the deleterious effect of dry heat on the lysine of proteins is that the free carboxyl groups of the dicarboxylic amino acids may react with the  $\epsilon$ -amino groups of lysine to form a new peptide linkage which would be resistant to enzymic digestion but not to acid hydrolysis.  $\epsilon$ -Amino peptides would, however, probably pass through the walls of the intestine and so appear in the nitrogen of the urine and not of the feces (*cf.* 15 for confirmatory evidence).

The results of this paper stress again the sensitivity of lysine in the protein molecule to relatively mild processing procedures.

We wish to thank Miss Margaret O'Shaughnessy of the Borden Diet Kitchen for preparing the foods tested in this experiment.

#### SUMMARY

A cake mix consisting of flour, sugar, egg white, lactalbumin, hydrogenated vegetable oil, dried yeast, molasses and salt with 25% of its calories as protein, had a very high protein efficiency (PE 3.3-3.5).

When this mixture was baked into a cake and the cake dried on the radiator, the PE decreased to 2.4. If the cake were dried in an oven overnight at 60°C., the P.E. decreased further to less than 1.5.

When the slices of cake were toasted in a low oven at 100-130°C., until they had the appearance of commercial rusk (Zwieback), the PE was less than 0.7; in some cases the animals were just able to maintain weight.

However, if 0.49% of *l*(-)-lysine were added to the oven-dried cake, the PE rose to 2.7; while the addition of 0.63% of lysine to the toasted material practically restored its initial nutritive value (PE 3.2).

The experiments indicate the sensitivity of lysine in the protein molecule to processing procedures.

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# The Reaction of Mustard Gas with Proteins. I. The Nutritional Value of Casein Reacted with Mustard Gas <sup>1</sup>

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## INTRODUCTION

Previous studies have shown that mustard gas (hereafter referred to as H) reacts with corneal tissue so rapidly that none of the free H which penetrates the cornea can be recovered after several minutes (1). More recent investigations in this and other laboratories have demonstrated that in skin, too, H reacts so quickly that therapy based on competing or decontaminating agents would be unsuccessful. While a part of the H reacts with the water in the tissue to form thiodiglycol, it now seems definite that the degree of injury produced by H in tissue closely parallels the amount of H which has become "fixed" (2). Accordingly, it may be assumed, as a working hypothesis, that if H could be split from its combination with tissue, it would be possible to reverse the process causing tissue injury. It will be recalled that following H burns there is a latent period of several hours before the clinical effects become evident and during this time the "fixed" H might be removed from combination with tissue, thus aborting the toxic process.

That skin, and probably other tissues, are incapable in themselves of decomposing at an appreciable rate the compounds resulting from the combination of H with tissue components follows from the work of Moritz and Henriques (2). These workers, using H containing radioactive sulfur, found that the amount of H fixed in the skin did not diminish significantly until it was sloughed off one month later as part of the scab. Furthermore, it is now well-known that the chemical binding between H and various substances is so firm that no appreciable

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Harvard University.

reversal occurs in the presence of compounds known to be highly reactive with free H.

The question arises as to what means compatible with cell life are available for removing the fixed H from the tissue components. The use of enzymes appears to represent one possible method of accomplishing the desired results.

The present investigation was designed to determine whether the enzymes found in the digestive tract of the rat or chick could degrade casein exposed to H sufficiently for the casein to be utilized for growth, and if not, to investigate the nature of the binding between H and casein by means of biological assays, using chicks and rats.

H-treated casein was prepared by exposing casein at pH 9.3 to a relatively large quantity of H, 56 molecules of H per molecule of casein. The product was precipitated at the isoelectric point, washed, redissolved with NaOH, reprecipitated, washed with increasing concentrations of alcohol and, finally, dried and ground to fine powder. The final product was practically odorless but, when redissolved in water, possessed the disagreeable odor characteristic of solutions in which H has hydrolyzed. The sulfur content averaged 3.5% which corresponded to approximately 30 H residues per molecule of casein.

Rats would not eat diets containing significant amounts of the casein prepared in this manner. Butyric acid, chocolate, sugar, *etc.*, were added in various combinations to the ration in an unsuccessful attempt to get them to eat the diet containing H-exposed casein.

Finally, it was found that sufficient amounts of the unpalatable compounds present in H-exposed casein could be removed by thorough treatment with activated charcoal so that the rats would eat adequate quantities.

### EXPERIMENTAL

Chicks, on the other hand, would eat casein reacted with H without the activated charcoal treatment. Hence, the first experiments were carried out with this species. The basal ration<sup>2</sup> was prepared and

<sup>2</sup> Basal Ration for chicks	Per cent
Sucrose.....	76
Gelatin.....	7
Yeast.....	5
Salt Mixture.....	5
CaHPO <sub>4</sub> .....	1
Corn Oil.....	5
Liver extract.....	1
Choline.....	0.1
Cystine.....	0.3

Water-soluble vitamins per 100 g. of ration; thiamin 200  $\gamma$ , riboflavin 400  $\gamma$ , calcium pantothenate 1500  $\gamma$ , nicotinic acid 2500  $\gamma$ .

Vitamins A and D fed by dropper twice weekly in excess of requirement.

supplements of normal casein or H-exposed casein were added at the expense of the sucrose. Two three-day old chicks were used for each of the experiments reported in Fig. 1. It will be seen that 5% normal casein was just sufficient to maintain weight, whereas, 10 and 15% normal casein resulted in progressively increased growth. When H-exposed casein was supplied at the 10% level no growth occurred. Moreover, the addition of 5% exposed casein to a ration containing 5% normal casein caused no significant increase in growth.

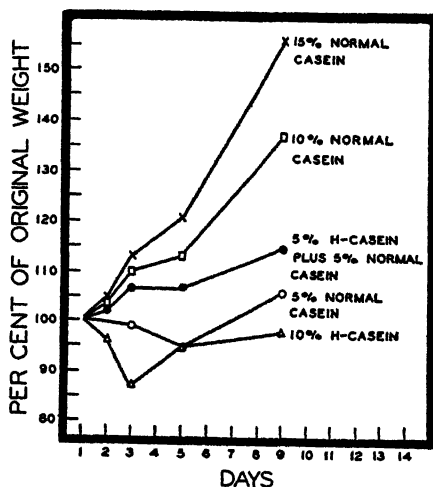


FIG. 1

Growth of Chicks on Basic Diet Plus Supplements as Indicated

A second series of experiments were performed in which chicks were fed rations containing charcoal-purified H-treated casein (Fig. 2). It will be seen that good growth resulted when the basal ration was supplemented with 10% normal casein. By contrast, when 10% H-exposed casein was added to the basal ration no growth occurred. To determine whether or not the H-exposed casein was actually toxic to the chicks, or was simply not assimilated, the 10% exposed casein was supplemented with 10% normal casein ration after 11 days (indicated by arrow). It is apparent from the good growth which resulted on addition of normal casein that the previous absence of growth was not a toxic manifestation.

A third ration, consisting of normal chick mash, was supplied to three chicks until the tenth day at which time the animals were placed on a basal ration supplemented with 10% H-treated casein. The line shows that excellent growth occurred up until the time the exposed casein was added, after which the animals began to lose weight.

All of the experiments are consistent with the belief that chicks are unable to utilize H-exposed casein. That the lack of growth was not due to failure to eat this substance was shown by observation of food consumption, and by the fact that growth was obtained when 10%

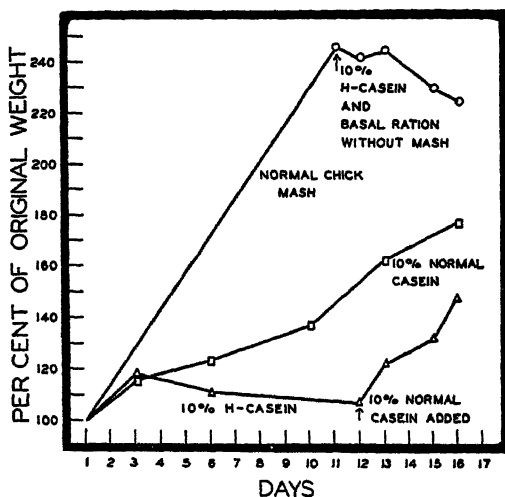


FIG. 2

Growth of Chicks on Basic Diet Plus Supplements as Indicated

normal casein was added to 10% treated casein. Chicks in the treated group which had received normal chick mash for ten days were eating well and consumed the treated casein ration in large amounts when first changed to this diet. Nevertheless, they also lost weight.

The basal ration supplied to the chicks contained approximately 12% protein and was adequate in arginine, lysine and cystine. Thus, it is probable that some amino acid other than these three was made unavailable by the H-treatment.

A basal diet<sup>3</sup> for 21-day old rats was prepared in such a way that essentially all of the protein was supplied in the form of casein. To test the adequacy of this ration preliminary experiments were carried out in which casein was supplied at three levels—6, 9 and 18%. The growth attained, when this diet was fed for a ten day period *ad lib.*, averaged 106, 135 and 173%, respectively, of the original weight. Eighteen per cent H-treated casein was then added to the basal ration and rats were placed on paired feeding with another group of animals receiving 18% casein which had been through the same process as the H-reacted material, except that no H was used.

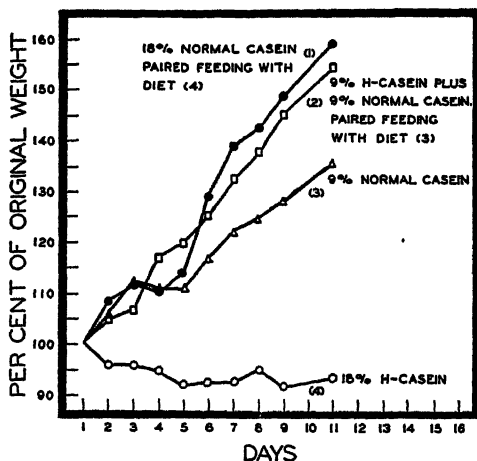


FIG. 3

Growth of Rats on Basic Diet Plus Supplement as Indicated

Fig. 3 shows that the rats on the treated material actually lost weight during the 10-day test period, whereas those receiving the same amount of control casein grew well. Moreover, it will be seen that the amount of growth of rats receiving 9% normal casein plus 9% H-casein was essentially the same as those supplied normal casein at the 18% level. Furthermore, when compared in paired feeding experiments with rats on 9% normal casein it appears that 9% H-casein has actually

<sup>3</sup> Basal diet for rats

Per cent

Starch.....	60
Corn Oil.....	10
Cod liver oil.....	2
Salt Mixture.....	4
Yeast.....	6



supplemented the 9% normal casein ration. The results illustrated in Fig. 4 would tend to confirm the latter interpretation. In these experiments the basal ration was supplemented with 6% control casein (filled circles) and the results compared with a similar diet in which 9% H-casein was given in addition. Again the rats which received H-treated material in addition to the normal casein showed a greater growth rate despite a greater consumption of food when the basal diet was supplemented with only 6% normal casein. These experiments are thought to indicate that partial utilization of H-casein occurred.

Failure of the H-casein to support growth in the absence of normal casein may be due to failure of the animals to split some essential amino

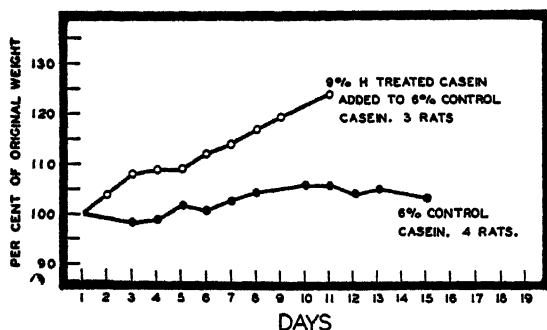


FIG. 4

Growth of Rats on Basic Diet Plus Supplement as Indicated

acids from their combination with H, to a remote effect on certain amino acids, or to a decreased rate of digestion of the whole H-casein molecule.

We wish to acknowledge the assistance of Drs. Frederick Stare and Mark Hegstead of the Division of Nutrition, Harvard Medical School, in carrying out the experiments on the chicks, and Phyllis Robison for technical assistance.

### SUMMARY

Exposure of casein to mustard gas renders the casein inadequate to support growth of chicks and rats when this material was fed as the source of most of the protein, although it did supplement normal casein when fed to rats. Lack of growth in chicks was not caused by an inadequacy of arginine, lysine or cystine.

No evidence of toxicity could be demonstrated when animals were fed H-exposed casein.

More detailed information on the question of the nature of the reaction of H with casein will be given in the paper which follows.

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# The Reaction of Mustard Gas with Proteins. II. Biological Assay of Amino Acids Affected <sup>1</sup>

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## INTRODUCTION

It was shown in the previous paper (1) that neither chicks nor rats would grow normally when fed diets containing casein exposed to mustard gas (hereafter referred to as H) as a chief source of protein. Since the H-treated casein was not toxic, the nutritional inadequacy was taken to indicate, either first, that the animals were unable to split the H residues ( $-C_2H_4SC_2H_4-$ ) from certain essential amino acids in the protein, or second, that reaction of casein with mustard rendered certain amino acids unavailable indirectly through some secondary effect on certain amino acids or by making the casein molecule indigestible as a whole.

## EXPERIMENTAL

If it could be shown that rats could split off H-residues from amino acids known to possess such groups it is apparent that the first explanation offered above could be ruled out. To determine this, two water soluble compounds, Semi-H valine <sup>2</sup> and Semi-H cysteine,<sup>3</sup> were prepared (2). The Semi-H derivatives, involving but one linkage with an amino acid were used instead of the H derivatives, where two linkages are present, in order to keep the experimental conditions as simple as possible. The assay was carried out by feeding three diets

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

<sup>2</sup> N-2-(2-hydroxyethylthio)-ethyl valine.

<sup>3</sup> S-2-(2-hydroxyethylthio)ethyl cysteine HCl.

containing the following: (1) all ten essential amino acids, including valine; (2) all essential amino acids except valine; and (3) all essential amino acids except valine, but with the addition of Semi-H valine equivalent in amount, on a nitrogen basis, to the valine.

Fig. 1 shows that the rats grew well on the control diet containing valine, but did not grow on the diet lacking valine or on the one with Semi-H valine. Animals grew well when valine was added to each of the latter diets.

Since cysteine can be synthesized by the rat from methionine, a somewhat more complicated experiment was necessary to test whether

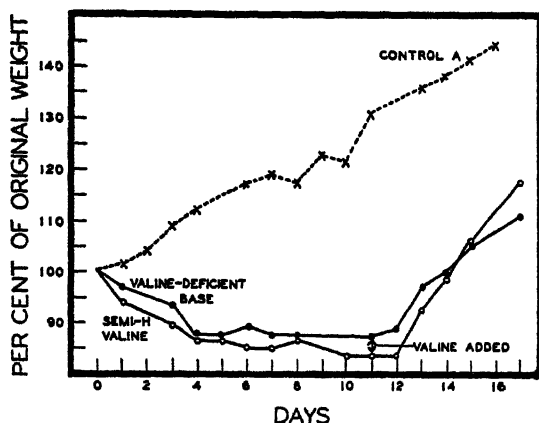


FIG. 1

Growth of rats on all essential amino acids (broken line); same diet without valine (filled circles); same diet without valine but plus Semi-H valine (open circles).

the rat could utilize cysteine from Semi-H cysteine. This consisted in decreasing the amount of methionine present in the diet until the growth rate of the rat became dependent upon the amount of cysteine available. The concentration of methionine found to be suitable for this purpose was 0.1%. In Fig. 2, it will be seen that, when the diet was cysteine free, the animal lost weight. When a rat was fed a similar diet except for the addition of 1% cysteine, growth was obtained. However, no growth was shown by rats fed the basal diet supplemented with Semi-H cysteine equivalent in nitrogen content to 1% cysteine. On the eleventh day a second unit of Semi-H cysteine was added to the Semi-H cysteine diet, and also to the diet containing no cysteine or

Semi-H cysteine, with no discernible effect. The difference in growth between the Semi-H cysteine diet and cysteine-free diet is of questionable significance.

It is concluded that rats cannot utilize either valine or cysteine from their Semi-H derivatives and presumably, therefore, cannot split off H residues from either the nitrogen of valine or the sulfur of cysteine.

Since rats could not utilize two typical amino acids in combination with H residues it now appeared worthwhile to attempt to determine the number of essential amino acids in the H-treated casein which had reacted with H.

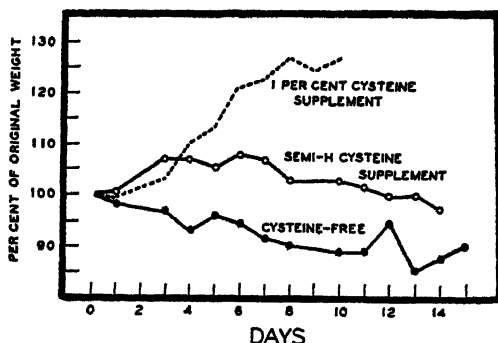


FIG. 2

Growth of rats on amino acid diet containing  $\frac{1}{2}$  minimum requirement of methionine but cysteine free (filled circles); the same diet supplemented with Semi-H cysteine (open circles); and supplemented with 1% cysteine (broken line).

The H-treated casein was assayed by feeding rats diets containing the protein to be tested supplemented with 9 of the 10 essential amino acids. Similar experiments were carried out using an acid hydrolysate of the H-treated casein and, in addition, the hydrolysate was assayed by means of a bacteriological method.

The first determinations were made by preparing 10 diets in such a way that each diet lacked but one essential amino acid. The amino acids in pure form were added to the base diet (1) at levels considered by Rose (3) to be necessary for growth. (See also Kinsey and Grant (4).) To each ration was then added 10% H-treated casein. Two additional diets served as controls, the first of which contained all ten amino acids, and the second, all ten amino acids plus 10% H-casein. One 21-day old rat was placed on each of the above diets for about ten days. In the instances where the rat did not grow the missing amino acid was added to the diet at the end of this period.

The results of these experiments are shown in Fig. 3. The arrow indicates the time at which the missing amino acid was added.

It will be seen that little growth occurred on the diet lacking histidine, and that none occurred on those lacking lysine, methionine or threonine. That the limiting factor was actually the amino acid in question is seen from the growth which occurred in every instance when the missing amino acid was added.

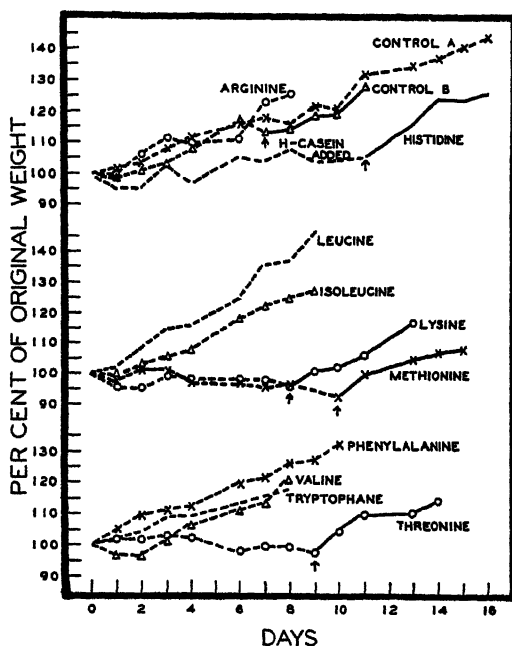


FIG. 3

Growth rate of rats on diets containing H casein supplemented with all essential amino acids except the one indicated. Arrow indicates addition of missing amino acid.

It is difficult from these experiments alone to be sure whether or not an actual mustard residue ( $-\text{CH}_2\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2-$ ) has become attached to some group of the amino acids, although the sulfur analyses of H-treated casein (3.5%) would support this contention. The fact that lysine has a reactive amino group not already covered in the protein molecule, and that both methionine and histidine have groups

which are known to react with H and are probably similarly available for reaction in the protein molecule, are suggestive that actual binding has occurred. However, the evidence available with regard to the probable degree of activity of the hydroxyl of threonine indicates that H would not be expected to bind with this group under the conditions used for exposing the casein.

To determine whether the alteration of the nutritive properties of these four essential amino acids was actually the result of a direct effect of H upon the individual acids, it was decided to hydrolyze the H-treated casein and again determine the growth-supporting capabilities of its constituents.

A hydrolysate of the H-casein was prepared by refluxing the protein preparation in 1:1 HCl for 24 hours, evaporating in vacuum to dryness, redissolving in water, re-evaporating to dryness, neutralizing with NaOH and re-drying. Four rations were prepared containing 10% hydrolysate plus the basal diet used previously (1), and the four acids under investigation, *i.e.*, methionine, threonine, histidine and lysine. As controls, three additional diets were prepared; one contained the base plus 18% normal casein; the second contained the base plus 18% control casein which had been put through the same procedure as used in preparation of H-casein; the third diet contained the base plus 18% of a hydrolysate of this control casein to which 0.2% tryptophane had been added.

The graphs of Fig. 4 show the weights of the rats supplied these rations plotted as *per cent* of their original weights.

Comparison of the growth curves for 18% normal casein with those for 18% control casein and its hydrolysate shows that the alkaline purification process in itself causes only relatively minor impairment of the nutritional value of casein.

Inspection of the growth curves of the rats on diets containing H-casein hydrolysate supplemented with all the essential amino acids except lysine, or methionine, or histidine, shows that no growth is obtained until these acids are added. The latter, therefore, appear to be available in inadequate amounts in the H-casein hydrolysate, a finding which confirms the results obtained by feeding the whole protein. By contrast, it will be observed that the growth obtained with H-casein hydrolysate in the absence of supplementary threonine was as good as that obtained when a threonine supplement was used. This result indicates that threonine must now be available in larger amounts than it was before the H-casein was hydrolyzed. While acid hydrolysis may have removed the H residue from threonine, for reasons cited



previously, it seems likely that some other mechanism is involved in determining the availability of this amino acid.

These results, viewed from the standpoint of our initial objective (1), namely, to determine whether the enzyme systems present in the digestive tract could split H residues from H-treated proteins, were entirely negative. However, the possibility existed that the reaction

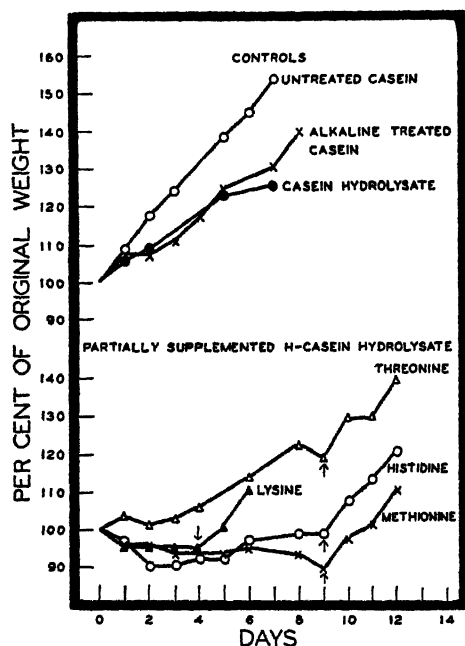


Fig. 4

Upper group (Controls all fed at 18% level.) Untreated casein (open circles); alkaline treated casein (crosses); alkaline treated casein hydrolysate (filled circles).

Lower group. H-casein hydrolysate supplemented with all essential amino acids except the one indicated. Arrow indicates addition of missing amino acid.

between H and casein at neutral pH might affect the availability of the amino acids differently than at 9.3 as was the case in the above experiments. To test this possibility H was reacted with casein at pH 7.4 using 52 molecules of H per molecule of protein. The pH was maintained by continuous titration. Using casein exposed to H at pH 7.4, at a 10% level, assays were carried out as before. In contrast to the

results noted previously rats failed to grow only on those diets lacking either methionine or threonine. The addition of these amino acids to the diet containing the H-treated casein was attended by good growth.

The animal assay method employed thus far is limited to testing the ten essential amino acids and is qualitative or, at best, roughly quantitative. Also, its practical use is confined to a few proteins, such as casein, the nutritive properties of which are well worked out. To test for possible effects of H on other amino acids of casein, to assay several other proteins, and also to try to obtain a more quantitative estimate of the binding, a bacteriological method for assaying amino acids was utilized. Two species of bacteria were used; *Lactobacillus arabinosus* and *Streptococcus lactis*. The former was used for all of the determinations except lysine and threonine.

The assays were carried out by the use of an adequate synthetic medium containing 16 amino acids. Media deficient in specific amino acids were made by simply omitting one of the amino acids from the mixture. Protein hydrolysates could then be tested semiquantitatively by adding them to the deficient medium in various amounts and comparing the resulting growth with that obtained from various concentrations of the amino acid.

No statement can be made as to the accuracy of the method in determining the absolute quantity of the amino acids present in either the untreated or treated proteins. However, repeated determinations of different samples of exposed casein show the same relative quantity of the amino acid and, in general, the control analyses are in fair agreement with the literature. The method in its current state of development appeared to be more satisfactory for some amino acids than others. Assays for arginine, glutamic acid, threonine and tyrosine were the least satisfactory. As in the animal assay work, results which indicate that amino acid in treated samples is present in normal amount, do not necessarily indicate that H has been without effect on the amino acid. This is true because the organism may be able to utilize an amino acid which has been affected or, as was found for threonine, the process of hydrolyzing the protein may in itself make available an amino acid which on other evidence appears to be affected by the H.

The percentage of apparently unaltered amino acids present in hydrolysates of casein treated with H in alkaline and in neutral solution, is shown in Table I. Values are included for a control which was

exposed to alkali in the same manner as was H casein during its preparation. The *per cent* composition of the protein is based on nitrogen content of the hydrolysate, using the factor 6.25.

It will be seen from the table that H treatment affects both methionine and lysine, the former apparently at both neutral and alkaline pH and the latter probably only in alkaline solution. These results are consistent with those found previously by the animal assay method.

TABLE I

*Per Cent of Amino Acids Present in Hydrolysates of Casein Treated with H at pH 9.3 and 7.4 Compared with Control Casein*

Amino Acid	H-Casein (Alkaline) 56:1 <sup>4</sup>	H-Casein (Neutral) 52:1 <sup>4</sup>	Control Casein
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Arginine	4.1	4.7	4.6
Glutamic acid	23.4	27.4	19.2
Isoleucine	6.8	6.5	6.5
Leucine	13.3	11.6	10.6
Lysine (Av. triplicate)	0.8	7.9	8.9
Methionine (Av. duplicate)	0.8	2.5	3.8
Phenylalanine	4.6	4.2	4.0
Threonine	4.8	3.6	3.0
Tyrosine	7.2	7.4	5.9
Valine	9.4	8.0	8.6

The biological importance of these findings depends to a large extent upon whether H combines with the same groups in other proteins, particularly those in living cells. With the object of determining to what extent the effect of H is the same on different proteins,

<sup>4</sup> Represents the ratio of molecules of H to molecules of protein (molecular weight 33,600) in the reaction mixture. Sulfur analysis indicates that 30 molecules of H have combined with the casein in alkaline solution, of which approximately 23 molecules can be accounted for by methionine and lysine.

<sup>5</sup> *Preparation of H-treated cornea.*

Beef corneas were immersed in kerosene containing 5 mg. of H/ml. and were allowed to remain there for a 24-hour period. This is sufficient to completely inhibit turgescence when subsequently immersed in water. The corneas were then washed in water, dried, ground and hydrolyzed.

H was reacted with corneal tissue<sup>5</sup> and with globulin and albumin<sup>6</sup> isolated from horse serum, by  $(\text{NH}_4)_2\text{SO}_4$  precipitation. The assay results for methionine and lysine are summarized in Table II.

TABLE II

*Effect of Mustard Exposure on the Methionine and Lysine Content of Globulin, Albumin and Cornea*

Amino Acid	H-Globulin	Control Globulin	H-Albumin	Control Albumin	H-Cornea	Control Cornea
Methionine	0.86	2.36	0.33	1.10	2.87	3.45
Lysine	6.30	9.26	1.03	1.31	6.07	6.07

It is apparent from the Table that exposure of globulin and albumin to mustard affects the methionine and lysine, the former to the greater extent, and that similar exposure of corneas to H affected methionine but not lysine.

Dr. Mark Hegstead of the Division of Nutrition, Harvard Medical School, carried out the bacteriological assays.

### SUMMARY

Solutions of casein exposed to mustard gas at pH 9.3 or 7.4 were prepared and assayed biologically to determine which of twelve amino acids tested (ten essential, tyrosine and glutamic acid) were made unavailable to support growth of rats or bacteria. When the reaction was carried out at pH 9.3 histidine, lysine, methionine and threonine were made unavailable for growth.

#### <sup>6</sup> *Treatment of globulin and albumin with H.*

200 ml. of solution containing 7.8 g. of globulin were reacted with 3.64 g. of H. The reaction was carried out in the presence of 4.2 g. of sodium bicarbonate which kept the pH at approximately 7.5. After allowing the reaction to go to completion, the solution was diluted with 400 ml. of water and 1600 ml. of isopropyl alcohol was added to precipitate the protein. The precipitate was collected by filtration, resuspended in methyl alcohol, refiltered and dried. A hydrochloric acid, 24-hour hydrolysate was prepared in the usual manner. On the basis of the hydrolysis products of H remaining in solution after reaction it is estimated that 132 molecules of H had combined per molecule of globulin, assuming a molecular weight for globulin of 150,000. H-treated albumin was prepared using the same weight of H per weight of albumin as was used for the globulin preparation. On the basis of a molecular weight of albumin of 67,000 it was calculated that 58 molecules of H had combined per molecule of albumin.

Assays of hydrolysates of mustard-exposed casein (pH 7.4) showed that histidine, lysine and methionine were still unavailable, but that threonine now became available for growth.

Rats were unable to utilize the cysteine or valine which had mustard residues attached to the sulfur and nitrogen of these amino acids, respectively.

The above results, along with sulfur analyses of the mustard-exposed casein, support the contention that mustard combines directly with the affected amino acids, excepting threonine, and that the nutritional inadequacy is due to inability on the part of the rat (or bacteria) to split off H-residues.

Exposure of horse serum globulin and albumin to mustard at neutral pH appeared to affect methionine and lysine. Similar treatment of corneal tissue showed slightly smaller quantities of methionine than did control corneas. The exposure had no effect on the lysine content.

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# On the Mechanism of the Catalase Inhibition by Anions

Kjell Agner and Hugo Theorell

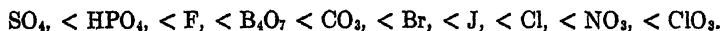
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Catalase was one of the ferments studied by Sørensen in 1909 when he demonstrated the dependence of ferment activity on hydrogen ion concentration. Catalase had its optimal effect in the vicinity of the neutral point and, with even relatively slight changes toward acid or alkaline reaction, he found a reduction in the activity. This is an observation that has since been confirmed by a number of authors.

Michaelis and Pechstein (2) showed that catalase was an amphoteric electrolyte and considered that the activity was due to the charge of the catalase molecule. They found a reciprocal relation between the effects of the anion and the hydrogen ion. At high hydrogen ion concentrations low anion concentrations were sufficient to provoke a certain inhibition and vice versa.

Various anions have a more or less strongly inhibiting effect upon the catalase activity. According to Michaelis and Pechstein sulphate ions inhibit only to a relatively slight extent, whereas chloride and, especially, acetate and nitrate ions are strong inhibitors. Santesson (3) examined several anions and gave the succession in the following way on a rising scale:



It was not yet known to these authors that the activity of the catalase is conditioned by its hemin, which was demonstrated by Zeile and Hellström (4), who also found that the inhibition of the activity by HCN and  $\text{H}_2\text{S}$  was accompanied by a shifting of the spectral bands. Keilin and Hartree (5) found analogous conditions as regards  $\text{NaN}_3$ ,  $\text{NH}_4\text{OH}$ ,  $\text{NaF}$ ,  $\text{NO}$  and  $\text{C}_2\text{H}_5\text{OOH}$ . The investigations of Pauling and co-workers (6) on the compounds of methemoglobin with some of these substances made it clear that the anions are bound to the iron atom in the hemin. Important preliminary work had been performed by Zeynek (7), Haurowitz (8), Lipmann (9) and Drabkin and Austin (10). As regards the lacto- and the horseradish peroxidase, Theorell and Paul (11) found that a hydroxyl group is bound to the iron atom in such a way that OH can be displaced by other negative ions, not only by the previously mentioned enzyme inhibitors (*e.g.*, fluoride) but also, *e.g.*, by phosphate ions in high concentration and at low pH.

Keilin and Hartree (5) noted a slight change in the position of the absorption band in the red on acidification of catalase solutions with sodium bisulphite, sulphur dioxide or acid buffers, which these authors ascribed entirely to the pH-change. Agner (12, 13), however, found that catalase solutions acidified to the same pH with acetate and phosphate buffers, respectively, showed different colors and different degrees of inhibition of the activity. In the present paper we have studied this phenomenon more closely by spectrophotometry and determinations of the activity, arriving at the conclusion that the iron atoms of the catalase are combined with hydroxyl groups which may be displaced not only by the previously known catalase inhibitors, but probably by all, at least low molecular, anions in sufficient concentration, in which connection the activity is inhibited to a corresponding degree.

### EXPERIMENTAL

In these experiments we have used both liver and erythrocyte catalase from horse, prepared according to earlier published methods (Agner 12, 13). The liver catalase was crystallized and had a value for Kat. f. 60–65,000, and an iron-content of 0.092%; 75% of the iron being bound in protohemin. The erythrocyte catalase had a value for Kat. f. 65,000–70,000.<sup>1</sup> The iron-content was 0.092%, and all the iron was bound in hemin. The preparations were electrodyalyzed until free from salts and quite clear. pH in the catalase solutions after dialysis was 5.55–5.60.

### OPTICAL DETERMINATIONS

The absorption measurements have been carried out with a photoelectric arrangement, on the principles described by Warburg and Negelein (14).

The degree of acidity of the catalase solution was adjusted to a definite pH value by addition of phosphoric acid. After this, buffer of the same pH was added in increasing amounts. The ion concentration was calculated and  $\log I_0 I \times \frac{1}{d}$  was determined and corrected for dilution.

#### *Experiments with Addition of Phosphoric Acid to Liver Catalase*

A. To 18.14 mg. of liver catalase in 6 ml. solution pH 6.10 was added 0.102 *m* phosphoric acid with simultaneous checking of pH. The changes in absorption were measured at 610  $m\mu$ . Thickness of layer 20 mm.

<sup>1</sup> The value Kat. f. = 100,000 for erythrocyte catalase previously given by Agner has not been found since, and was thus probably too high.

TABLE I A

ml. phosphoric acid 0.102 <i>m</i>	pH	-log [H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]	log I <sub>0</sub> /I × $\frac{1}{d}$ corr. for dilution
—	6.10	—	0.295
0.015	4.60	3.60	0.300
0.022	4.18	3.44	0.302
0.027	3.96	3.35	0.303
0.052	3.43	3.08	0.312
0.072	3.29	2.95	0.322

B. After adjusting the pH of the solution to 3.29, 3 *m* NaH<sub>2</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub>-buffer was added. Diluted 100 times, the buffer showed pH 3.30.  
λ = 610 mμ.

TABLE I B

-log [H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]	log I <sub>0</sub> /I × $\frac{1}{d}$ corr. for dilution	pH control
2.95	0.322	3.29
2.65	0.325	—
2.35	0.330	—
2.05	0.338	—
1.75	0.350	—
1.46	0.365	—
1.16	0.380	3.29
0.87	0.394	—
0.58	0.399	—
0.32	0.400	3.03

From experiment I A it is shown that light absorption at 610 mμ increases through the pH-change from pH 4 to pH 3. That this change is conditioned by the pH value and not by the phosphate concentration is clear from experiment I B. H<sub>2</sub>PO<sub>4</sub><sup>-</sup> can, certainly, combine with Fe, but not to any appreciable extent until higher concentrations are reached than those which existed in I A. The phosphate ion is bound approximately according to a theoretical, simple dissociation curve (pK'<sub>FeH<sub>2</sub>PO<sub>4</sub><sup>-</sup></sub> = 1.54). The deviation at the highest phosphate concentrations is probably due to the slight sinking of the pH-value.

#### *Spectrophotometric Experiments with Different Anions*

Acetate buffers of different pH were added to a solution containing 18.14 mg. liver catalase in 6 ml., the pH in each experimental series



being kept constant. The light absorption was measured at 610 and 580 m $\mu$ .,  $d = 20$  mm. Two series, at pH 5.61 and 4.72, respectively, are shown in Fig. 2.

The experimental results in Fig. 2 agree well with the theoretical, simple dissociation curves. The deviations at the highest acetate concentrations are probably due to slight cloudiness in the solution resulting from the high salt concentration.

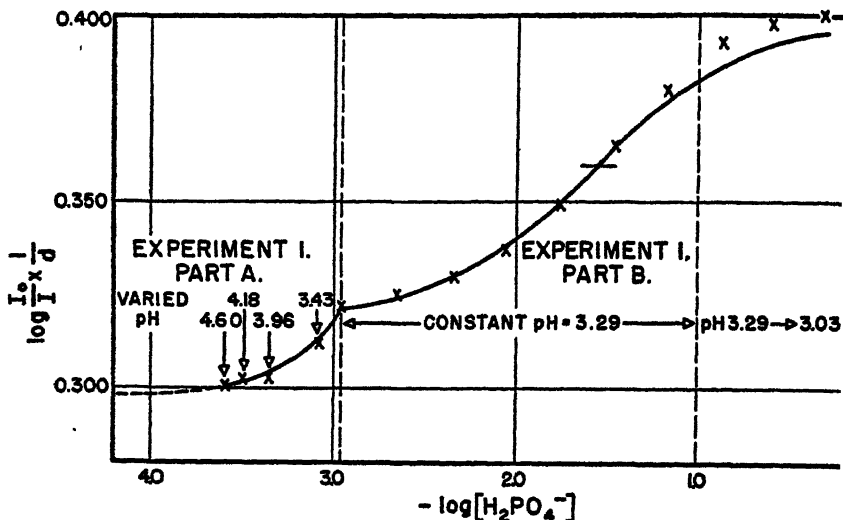


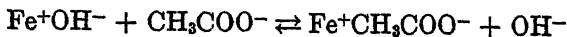
FIG. 1

Light Absorption of Liver Catalase at Varied pH and  $H_2PO_4^-$ -Concentration

The curve in part B is calculated:  $pK'_{Fe}; H_2PO_4^- = 1.54$

All the results of measurements at different pH are given in Table II. The value for  $-\log [CH_3COO^-]$ , when half the change in the light absorption had taken place, is referred to as  $pK_{app(arent)}$ .

Table II shows that, between pH 3.81 and 5.61, the dissociation constant for the binding of the acetate to Fe is dependent upon pH. This is explained by the assumption that a hydroxyl ion is bound to this catalase hemin-Fe. The acetate ion competes with the hydroxyl, thus



The real constant

$$K = \frac{[Fe^+][OH^-]}{[Fe^+OH^-]}$$

can certainly be calculated from this experimental series, but as the value would be based entirely on the determination at pH 3.55 we

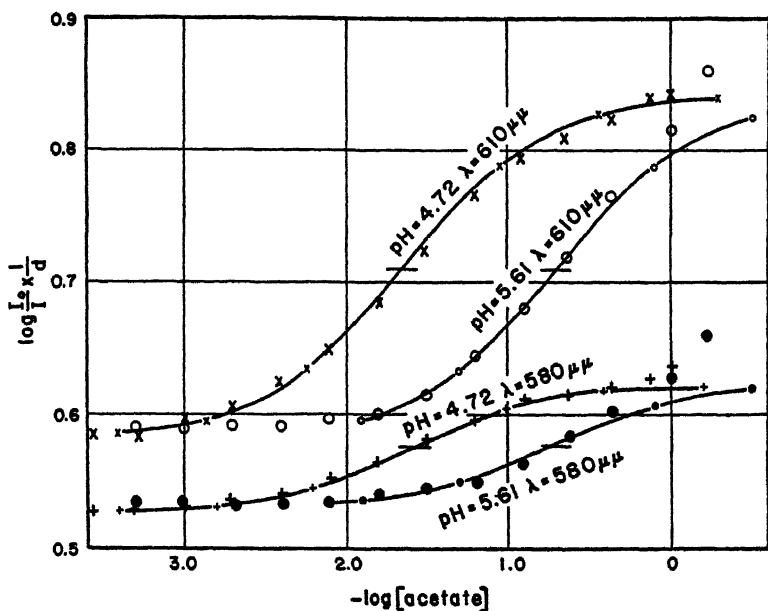


FIG. 2

#### Light Absorption of Liver Catalase at Varied Acetate Concentrations

The points are experimental, the curves theoretically calculated on the assumption that  $pK'$  is 0.70 at pH 5.61 and 1.6 at pH 4.72.

TABLE II

pH	$pK_{app}$	$\Delta$ pH	$\Delta$ $pK_{app}$
3.55	2.41		
3.81	2.40	0.26	0.01
4.14	2.08	0.33	0.32
4.45	1.84	0.31	0.24
4.72	1.63	0.27	0.21
5.61	0.66	0.89	0.93

have refrained from making this calculation. As will be shown below, an approximative value can be obtained from the determinations of activity in the presence of acetate (p. 334).

In a similar way the change in light absorption was determined at 610 and 540  $m\mu$ , when sodium formate was added in increasing amounts.

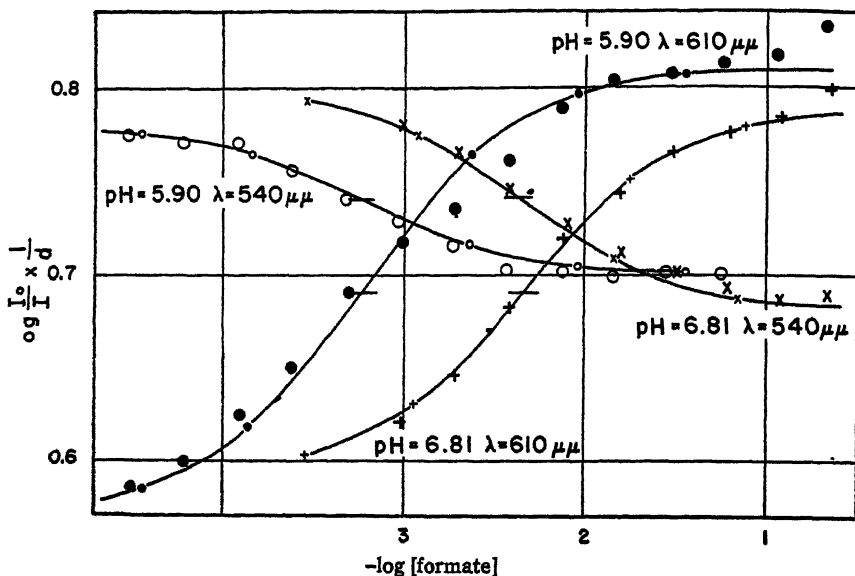


FIG. 3

Light Absorption of Liver Catalase at Varied Formate Concentrations

18.14 mg. of liver catalase in 6 ml. *m*/100 phosphate buffer. pH was 5.91 in one series, 6.81 in another. *d* = 20 mm.

As was the case on addition of acetate, simple dissociation curves were obtained. The points are experimental, the curves theoretically calculated on the assumption that  $pK'_{Fe^+, HCOO^-(app.)} = 3.24$  at pH = 5.91 and 2.34 at pH 6.81. As may be seen from the figure, the values obtained agree well with the assumption. A change of the pH by 0.90 gives an equally large change of  $pK'_{app.}$  within this pH-range, which further supports the view that a hydroxyl group is dissociably bound to the iron atom that reacts with the formate ion.

The formate ion has an affinity for the catalase-Fe which is about 800 times greater than that of the acetate ion.

We have tried to exploit the greater affinity of the formate to catalase-Fe for the spectrophotometric titration of catalase under favorable conditions, *i.e.*, low pH and high catalase concentration. Under these conditions the small amounts of formate added should, as long as Fe is in sufficient excess, be practically completely bound to the catalase.

Experiments also showed that this was the case within the pH-range 3.3–4.0, inasmuch as the light absorption at first rose in direct proportion to the addition of formate. It was obvious that the apparent dissociation constant between Fe and formate diminished toward the acid side; but to calculate definite values was impossible owing to the great affinity of the formate for Fe at these pH-values.

Orientating experiments showed that the lactate, propionate, chloride and sulphate ions combine in an analogous way with catalase-Fe.

Fluoride gave greater changes in the light absorption than the other anions. Fig. 4 shows the molar absorption coefficient,  $\beta$ , for liver catalase and erythrocyte catalase at various fluoride concentrations, constant pH = 4.86,  $\lambda = 6.10 \text{ m}\mu$ .

The molar light absorption is greater for erythrocyte than for liver catalase. As the former contains 4 protohemin groups and the latter 3 protohemin groups and 1 verdohemin group per molecule, this thus means that the light absorption of the verdohemin at  $610 \text{ m}\mu$  is lower than that of the protohemin. If the values for  $3/4$  of the  $\beta$  of the erythrocyte catalase at various fluoride concentrations are calculated, these are found to lie  $0.4 \times 10^7$  lower than those for the liver catalase right up to  $\text{pF} = 1.6$ , corresponding to  $m/40$  fluoride. This is probably due to the fact that the liver catalase contains one molecule of verdohemin. At higher fluoride concentrations  $\beta$  for the liver catalase begins to decrease, and approaches  $3/4$  that of the erythrocyte catalase, which phenomenon recurred constantly in several series of experiments at different pH. This can scarcely be due to any other cause than the fact that, at high fluoride concentrations, the verdohemin gives a fluoride compound with lower light absorption. The erythrocyte catalase, which lacks verdohemin, does not give any corresponding reduction of  $\beta$  at  $610 \text{ m}\mu$ .

#### ACTIVITY DETERMINATIONS

The reaction constant for the decomposition of hydrogen peroxide by catalase is determined by titrating the residual amount of

hydrogen peroxide in samples, taken as a rule 3, 6 and 9 minutes after the beginning of the reaction. From the values obtained, the reaction constant is calculated. This method has the disadvantage that the reaction constant decreases markedly during 9 minutes, so

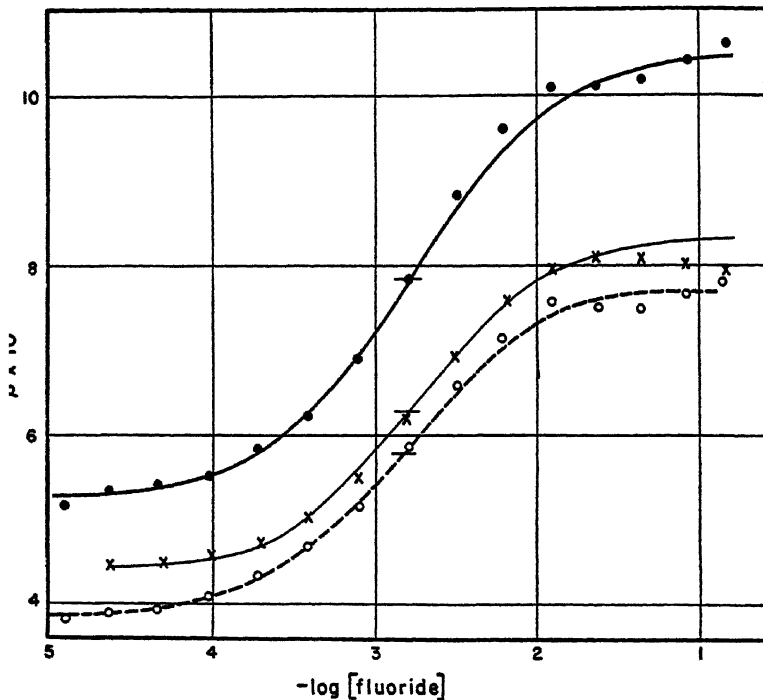


FIG. 4

Molar Absorption for Liver Catalase (X) and Erythrocyte Catalase (●)  
at pH = 4.86 and Varied Fluoride Concentrations

The points (○) are calculated as 3/4 of the absorption coefficient values for erythrocyte catalase.  $\lambda = 610$ . Curves theoretically calculated, points experimental.

that the 3 minute period gives a higher value than the 6 minute period and the 6 minute period a higher value than the 9 minute period. This is considered due to gradual destruction of the catalase. The values for Kat. f. given in the literature have generally been obtained by extrapolating the reaction constant to the time = 0 min. As this

method is rather unsatisfactory, we took the samples after 8 and 10 minutes instead, when values agreed so closely that one could take the mean. So much catalase was used that about half the hydrogen peroxide was decomposed during this time. With this method the values for Kat. f. are, of course, lower than when extrapolation is resorted to. In the experiment described below, however, this was of subordinate importance, as all the values for the reaction constant were expressed as a percentage of the value obtained with the same catalase solution in *m*/50 phosphate, pH 6.81 and hydrogen peroxide. The temperature was kept constant with a water thermostat at, in general, 20°C., in some experiments 0.8°C.

The reaction flasks, which should all be of the same size and shape—as catalase is to a certain extent adsorbed to glass walls—were cleaned between each experiment with bichromate-sulphuric acid and repeated rinsings with distilled water. All reaction solutions were prepared with water redistilled in glass vessels.

Altogether, about 2,000 determinations of activity were carried out in connection with the investigation.

#### *Experiments at Various Hydrogen Ion Concentrations*

In order to be able to study the inhibiting effect of different anions in different concentrations and at different pH-values, it was necessary, as in the optical experiments, to use an acid by means of which the hydrogen concentration in the reaction mixture could be adjusted to the desired value down to the stability limit for catalase, pH 3.2, without the anions of this acid noticeably combining with the catalase iron. For this purpose, in the determinations of activity, hippuric acid was used, which, in the experiments performed, proved to have the desired property. It behaved in this respect analogously with phosphoric acid, but had the advantage of exercising, because of its dissociation constant =  $10^{-3.81}$  (Josephson, 15), a buffer effect at least at the more acid of the pH-values at which the determinations of activity were carried out. The catalase activity was determined in three different series, at pH 3.3, 3.6 and 3.9, with rising hippurate concentration where the weakest solution only contained the amount of hippuric acid required for acidification to the respective pH. The results are shown in Fig. 5.

As may be seen from the figure, the curves are flat at the lower hippurate concentrations. It is only at considerably higher hippurate

concentrations than those which must be used for acidification of the solution, that anion inhibition occurs.

A large number of determinations, reproduced in Fig. 6, show that the catalase activity decreases acid medium. The effect is

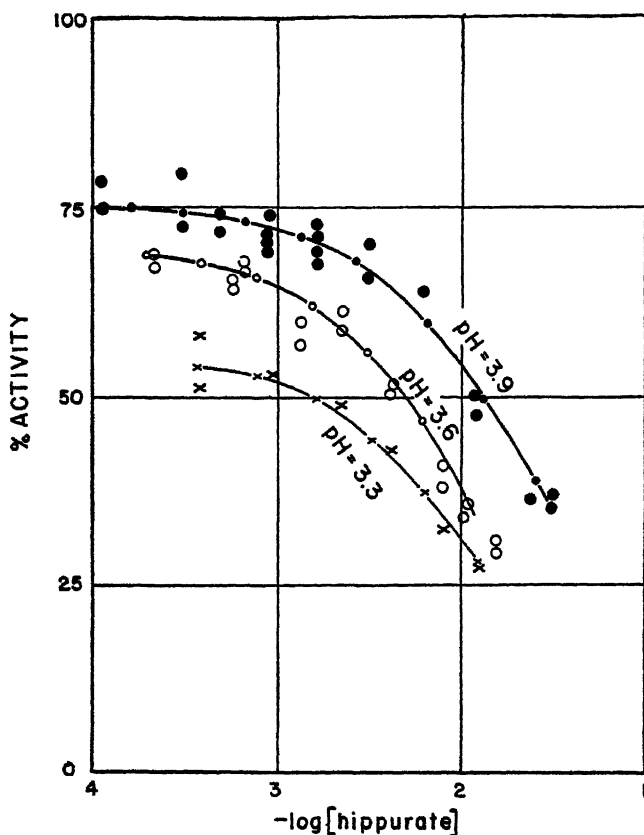


FIG. 5

Percentage Catalase Activity in Hippurate Buffers at pH 3.3, 3.6 and 3.9

markedly less, however, than that found by earlier workers (1, 2), which is obviously due to the fact that in those experiments a considerable anion inhibition was added to the pure pH-effect. The activity does not decrease, however, according to a simple dissociation curve, but in two phases. The form of the curve, within the wide margins of

error, agrees with that which is obtained if one superposes one simple dissociation curve on another, the one with  $pK = 4.90$  and ranging between 100 and 75% activity, the other with  $pK = 2.75$  and ranging between 75 and 25% activity.

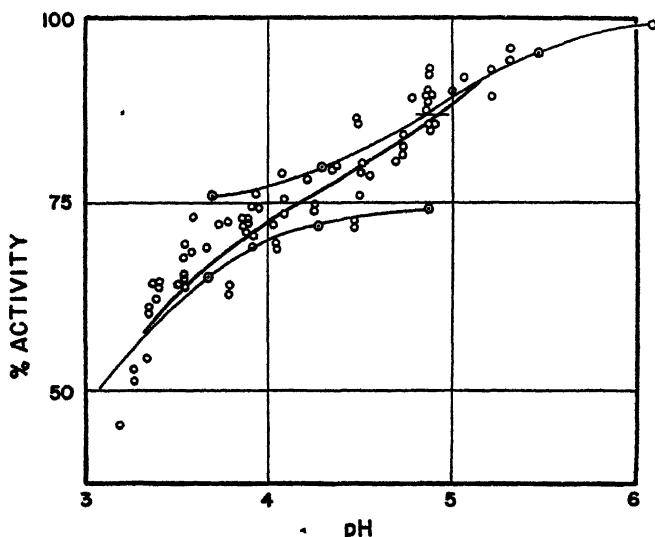


FIG. 6

The Effect of pH of Solution on Activity of Liver Catalase

### *Experiments with Acetate*

In the same manner as that described in connection with the experiments with hippuric acid/sodium hippurate mixtures, the activity for horse liver catalase was determined in serial experiments at constant pH and with rising acetate concentration in the solutions. These were prepared by diluting an acetate buffer of definite acetate ion concentration and pH with hippuric acid solution of the same pH. The dilution was carried out in such a way that the acetate ion concentration in one flask and that in the following one always stood in the ratio of 2:1. In Fig. 7 the percentage values for the activity at various acetate concentrations as compared with the catalase effect in phosphate buffer pH 6.8 are indicated.

The lines connecting the different values have not the form of the simple dissociation curve. To make the values found in the activity



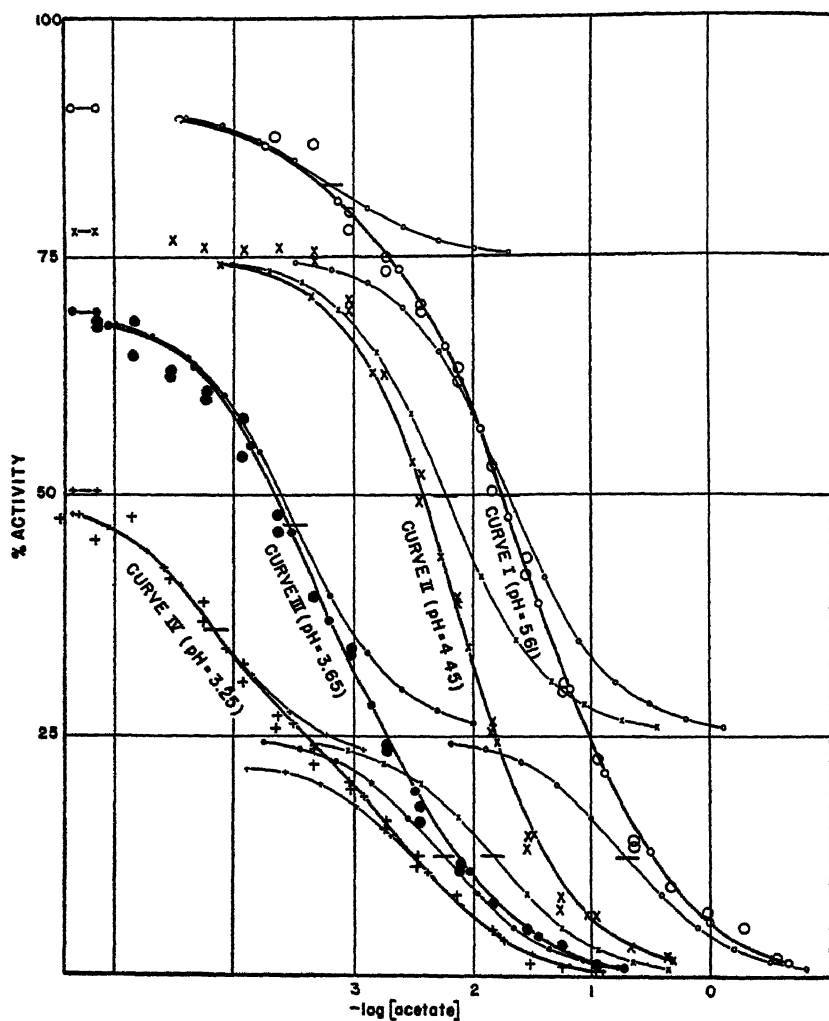


FIG. 7

Liver Catalase Activity at Varied pH and Acetate Concentrations

	pH 5.61	pH 4.45	pH 3.65	pH 3.25
Experimental points	○	X	●	+
Superposed curve	○—○	X—X	●—●	+—+
Partial curves	○—○	X—X	●—●	+—+

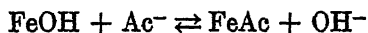
determinations conform with a theory which also includes the spectrophotometric results, one must resort to the rather unattractive hypothesis that the different hemin parts of the catalase molecule have different properties both spectrophotometrically and with respect to their individual contributions to the total activity, as well as regarding the effect of the hydrogen ion concentration on their activity. Such complicated conditions, of course, can not be elucidated mathematically or graphically on the basis of the hitherto existing experimental material. In Fig. 7, however, we have shown the curves found experimentally as superposed results of simple dissociation curves. The partial curves which lie between 0 and 25% activity correspond to the values found spectrophotometrically in experiments involving addition of acetate. The partial curve within the region 75–90% activity corresponds to one of the dissociation constants ( $pK = 4.9$ ) which has been shown as a pure pH-effect in the experiments with pure hippuric acid (Fig. 6). The values within the region 25–75% activity we were obliged to fit in empirically. Such graphic speculations clearly have only a limited value and can, at best, yield working hypotheses for continued investigation.

An important point, however, is the fact that the experiments on the inhibition of activity with acetate gave further support to the assumption that the active catalase iron is dissociably combined with hydroxyl. One can get an idea of the dissociation constant

$$K = \frac{[Fe^+] \times [OH^-]}{[Fe^+OH^-]}$$

from the following method of calculation, which, as regards horse radish and the lactoperoxidases, where conditions are simpler, gave accurate results (11).

In Fig. 8 we have indicated the  $pK_{app}$  value at different pH both for the spectrophotometric experiments and for the partial curves within the region 0–25%. At higher pH the connecting line between the points runs at an angle of  $45^\circ$ , while at lower pH it approaches  $pK_{app} = 2.5$  asymptotically. This observation can only be interpreted to mean that the iron atom of the hemin has a dissociably bound hydroxyl group and that, on acid reaction, the hydroxyl ion more easily dissociates, when it can be replaced by competing acetate ions.



The real constant for the dissociation  $\text{FeOH} \rightleftharpoons \text{Fe}^+ + \text{OH}^-$  has been calculated by means of the formula

$$K_{\text{Fe,OH}} = \frac{K_{\text{Fe,Ac}} \times [\text{OH}]}{K_{\text{app.}} - K_{\text{Fe,Ac}}}$$

which may be derived for the case in which two ions, in this case  $\text{OH}^-$  and  $\text{Ac}^-$ , compete for a bond to the same atom. According to Fig. 8, the curve approaches the value 2.5 asymptotically. The dissociation constant  $K_{\text{Fe,Ac}}$  thus becomes  $= 10^{-2.5}$ . As the mean of seven calculations the constant  $K_{\text{Fe,OH}}$  becomes:  $10^{-10.2}$ .

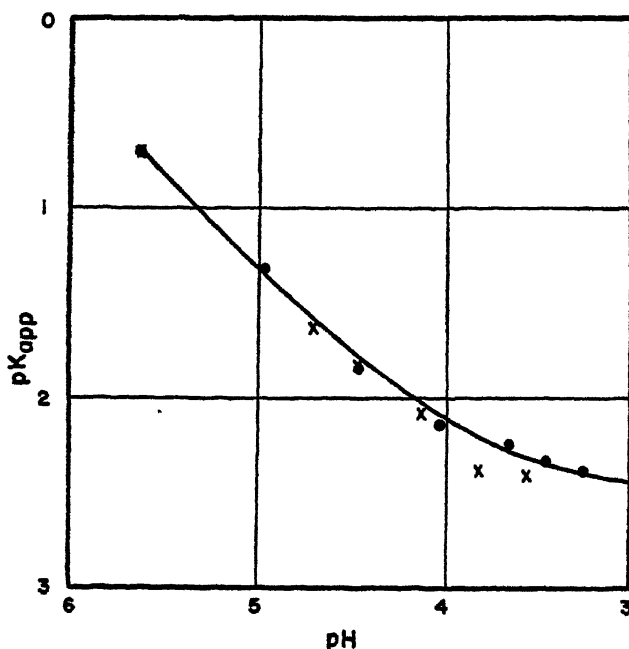


FIG. 8

Liver Catalase in Acetate.  $pK_{\text{app}}$  Plotted Against pH

- Values from determinations of activity (partial curve 0-25% activity).
- × Values from determinations of light absorption.

### *Experiments with Formate*

The activity of both horse-liver and horse-erythrocyte catalase has been determined in formate solutions in the same manner as that

described for the inhibition experiments with acetate. The determinations have been carried out at both 20°C and 0.8°C. The activity has been expressed as a percentage of the reaction constant in phosphate solutions pH 6.8 *m*/100 of the same temp. as the sample.

The results are shown in the figure below.

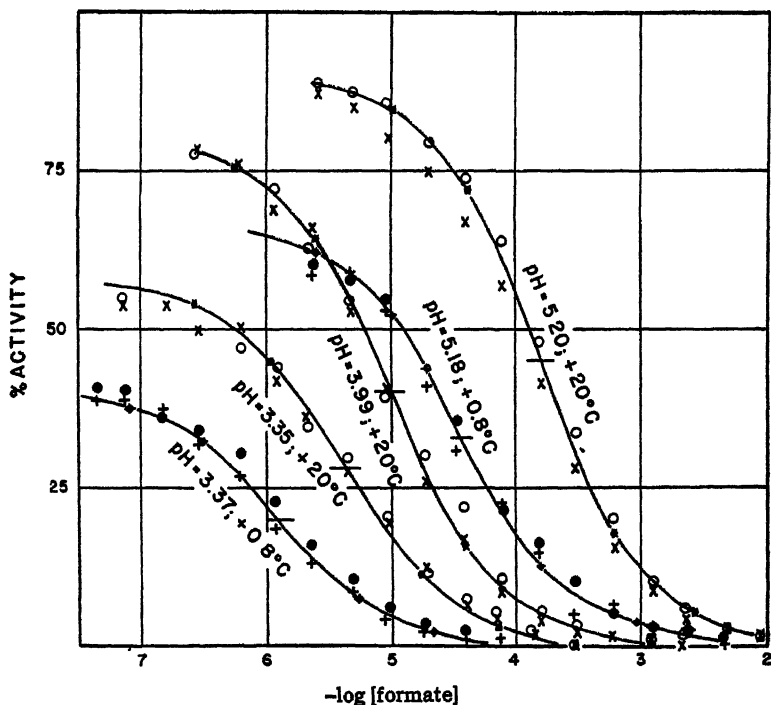


FIG. 9

Erythrocyte and Liver Catalase at Varied pH and Formate Concentrations

- |                                |                          |
|--------------------------------|--------------------------|
| ○ Erythrocyte catalase, 20°C.  | × Liver catalase, 20°C.  |
| ● Erythrocyte catalase, 0.8°C. | + Liver catalase, 0.8°C. |

The experimental results in the different series closely coincide with calculated dissociation curves for a monovalent electrolyte. This is in opposition to what was observed in the experiments with acetate buffers. The experiments with liver and erythrocyte catalase plus formate gave results in close agreement with each other.

In Fig. 10 the  $pK_{app}$  values at various pH have been indicated both for the spectrophotometric experiments and the activity curves. At higher pH the connecting line between the points runs at an angle of  $45^\circ$ , while at lower pH it diverges from this direction. As in the case of the experiments with acetate, we consider that this is probably due to a dissociation of an  $OH^-$  from the Fe-atom of the catalase on acid reaction. The spectrophotometrically determined

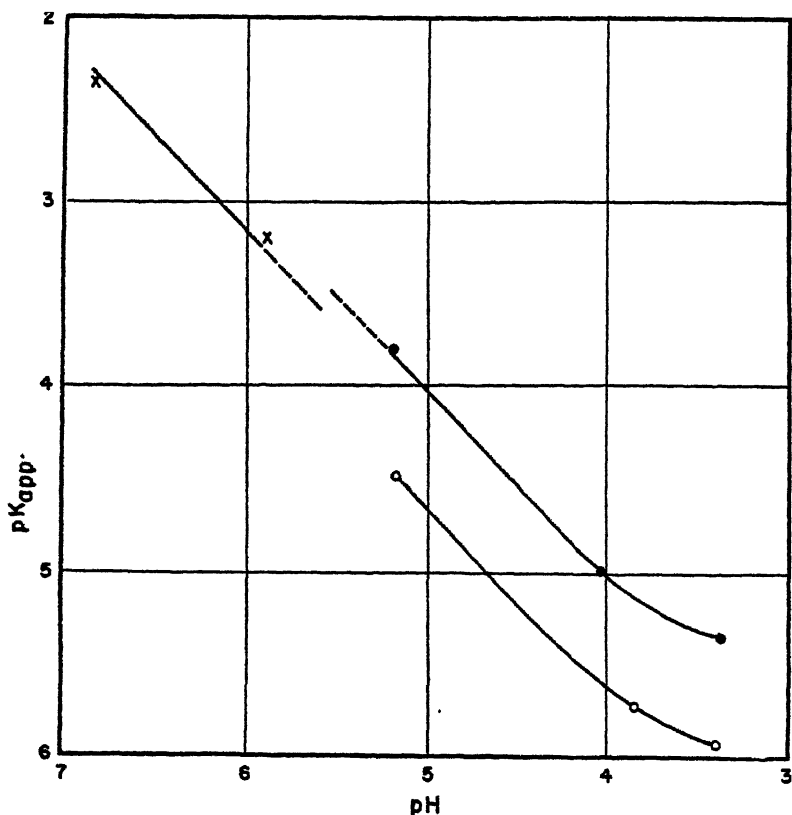


FIG. 10

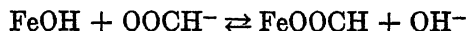
Catalase in Formate.  $pK_{app}$  Plotted Against pH

- × Values from determinations of light absorption
- Values from determinations of activity 20°C.
- Values from determinations of activity 0.8°C.

$pK_{app}$  values deviate only insignificantly from the points obtained from the activity measurements.

The determinations of the catalase activity carried out at 0.8°C. also agree with common dissociation curves. The activity at this temperature, however, was considerably lower, while the value for  $pK_{app}$ . Fe, formate is, at the same pH, 0.65 units higher than at 20°C.

The heat of ionization for the reaction



can, from the data found, be calculated from the formula

$$Q = \frac{4.571(\log K_2 - \log K_1)T_1 \times T_2}{T_2 - T_1},$$

whence  $Q = +12,400$  cal./mol. This means, in other words, that the difference between the heat of ionization for Fe-OH and for Fe-OOH in catalase is 12,400 cal.

### SUMMARY

1. Spectrophotometric measurements and activity determinations have been carried out on preparations of horse liver and horse blood catalase in the presence of various anions and at various pH-values.

2. The results show that the hemin iron is dissociably combined with a hydroxyl group which seems to be necessary for the catalase activity. This  $OH^-$  can be displaced by all the anions investigated, in which connection changes in the light absorption and an inhibition of activity that, at least in certain cases, runs parallel therewith have been demonstrated. Older investigations on anion inhibition of the catalase effect are herewith explained. A particularly strong inhibitor is formic acid, which is 800 times stronger in this respect than acetic acid. Acetic acid gives difficultly explainable discrepancies between spectrophotometric and activity determinations.

3. It has been possible to determine the pure pH-effect upon the catalase activity, and it has been found to be far less than has been assumed by previous investigators; what the latter have considered to be a pH-effect has been mainly constituted by an anion inhibition which increases as the pH falls.

4. Some values for the dissociation constants for the compounds of the active catalase iron with hydroxyl and other anions are given.

The difference in the heat of ionization between iron hydroxyl and iron formate compounds in catalase is 12,400 cal.

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## Book Reviews

**Microbes.** De la naissance et de la vie de quelques découvertes illustres en microbiologie. By PAUL HAUDUROY. F. Rouge & Cie S.A., Lausanne, 1944. 138 pp. Price: Swiss francs 5.50.

The book is a popular account of three great discoveries, Jenner's discovery of vaccination and Pasteur's discoveries of the anthrax and rabies vaccines. Written in a passionate and oratorical style the book dramatizes and glorifies the part played by the individual genius without inquiring into his background. It does not present any new facts nor any new interpretations of known facts. This is not intended in a popular book but the facts should be correct and this book unfortunately contains a good many mistakes of which I would like to point out only a few. There is no doubt that smallpox was a great killer but it certainly is exaggerated to say (p. 22) that everybody or almost everybody had the disease and that fifty, sixty, or seventy per cent of the patients died. If this had been the case the European population would have been wiped out very soon. The first vaccinations in Vienna were performed not by Jean de Carro (p. 60) but by Pascal Joseph Ferro. This does not detract from the great credit due to Carro who undoubtedly was the most active propagator of vaccination on the Continent and was held in high esteem by Jenner. The *Bibliothèque Britannique* published in Geneva through which vaccination became known on the Continent was not "un curieux journal" (p. 59) but an interesting attempt to oppose British liberalism to the radicalism of the French revolution.

The second shorter part of the book, on Pasteur, is much more correct because it follows René Valléry-Radot's biography. The illustrations are good and well selected. A number of disturbing printing errors have crept in (*e.g.* p. 52, Farguhar for Farquhar; p. 49, countries for counties; or, p. 52, the vaccine institute being founded in 1779 long before the discovery of vaccination).

The book makes one sadly realize that the early history of vaccination still has to be written. John Baron's *Life of Jenner* (London, 1838) was a glorification of Jenner and Charles Creighton's, *Jenner and Vaccination* (London, 1889) was an attempt to debunk Jenner that went much too far. The Johns Hopkins Institute of the History of Medicine, which probably possesses the best collection of literature on vaccination, will publish in the near future a collection of manuscript documents, several hundred letters of Jenner, Jean de Carro and others, that will throw some new light on the subject.

HENRY E. SIGERIST, Baltimore, Md.

**Gene Action in Micro-organisms.**—A Conference including papers by C. C. LINDEGREEN, E. L. TATUM and G. W. BEADLE, M. DEMEREK, S. SPIEGELMAN, A. HOLLAENDER, J. P. GREENSTEIN and H. W. CHALKLEY, J. W. GOWEN, T. M. SONNEBORN, M. DELBRÜCK, S. E. LURIA and S. EMERSON. *Annals of the Missouri Botanical Garden*, XXXII, No. 2, April, 1945. A special number published at



Galesburg, Illinois by the Trustees of the Missouri Botanical Garden, St. Louis, Mo., 1945. 156 pp. Price \$5.00.

Although the titles of most of the eleven papers in this publication indicate a principal interest in genetics, one can hardly over-rate the importance of the subject matter to the future development of biochemistry. During the many decades that have passed since the beginnings of the science of biochemistry, there has been accumulated a tremendous store of knowledge concerning the chemical composition of living cells. Furthermore, in recent years, a chemical basis has been established for understanding the mechanisms of many of the multitudinous dynamic changes that occur during life processes. A newer development, which brings together various divisions of science as "biochemical genetics," shows great promise in the achievement of a deeper understanding concerned with the problems of how living cells carry out specific chemical reactions, how the abilities to carry out these reactions are transmitted from one generation to the next, and what chemical reactions make one organism different from another. At the present time there seems little need for rationalizing the use of microorganisms for the purpose of attacking these problems, since certain of the fundamental principles of genetic transmission of biochemical abilities appear to be the same for all living forms.

A consideration of the papers under discussion leads to a general impression on the mechanisms of control and perpetuation of biochemical reactions. Each living cell contains units of nucleoprotein with specific chemical configurations. These units or genes are usually carried in characteristic nuclear structures (chromosomes). During simple cell divisions the chromosomes reproduce themselves to give each new nucleus a full complement of the genes carried by the parent. According to the conclusions reiterated in the paper by Tatum and Beadle a gene has, in addition to the power of self-duplication, the ability to determine the final chemical configuration of one enzyme. In turn, this enzyme acts as a catalyst for one specific chemical reaction. Though such a description is considered to be too simple by a number of workers, it is well supported by experimental work on the mold *Neurospora*. Many experiments on other organisms indicate that these principles form the basis for a broad generalization, but there are numerous cases that will require analysis in terms of specific chemical changes before such a generalization will stand unquestioned.

Although the *Neurospora* work has been concerned with genes and with the corresponding chemical reactions, the mediating enzymes have not been considered from the experimental standpoint. Since all enzymes do not act specifically on one substrate, it should be appropriate to consider that a gene controls a type reaction rather than one reaction.

Experimental work described in three of the papers of the symposium (Sonneborn, Spiegelman, Lindegren) seems to necessitate the introduction of a significant modification into the scheme of biochemical inheritance discussed so far. Convincing experiments by Sonneborn on the organism *Paramecium aurelia* indicate that a certain gene consists of two parts. One component (K) has a definite chromosomal locus and is inherited in a Mendelian fashion. The second component (*kappa*) can be transmitted through the cytoplasm, but a nuclear combination of K and *kappa* appears to be necessary for the reproduction of *kappa*. An extreme view taken by

Spiegelman and by Lindegren suggests that gene-like units that control the fermentation of galactose and of melibiose in yeast can be inherited entirely through cytoplasmic transfer. Although the existence of these units is not unreasonable, the implications arising are so great that a rigorous proof is necessitated. According to Spiegelman, the findings can be interpreted in a more conventional fashion but "such theorizations will not be 'pleasingly simple.'"

An excellent graphic description of some possible mechanisms of gene duplication and enzyme formation is presented in a paper by Emerson. Though these mechanisms have a sound basis in enzyme and immunochemistry their verification has largely eluded experimental proof.

Two papers concerned with the interrelations between host and virus (Gowen, Luria) represent a most interesting trend in biochemical genetics. It is demonstrated that viruses can be caused to mutate to have different degrees of virulence without loss of powers of self-duplication and in some cases without a measurable alteration of antigenic properties. These changes are perhaps comparable to the apparent abilities of some genes to mutate to quantitatively different levels of activity without loss of the power of self-duplication and the potentiality for back mutation. Mutations of bacteria to strains having new growth factor requirements, as correlated with virus resistance, is an item of special interest (Luria).

The symposium includes discussions of spontaneous mutation rates of bacteria (Delbruck), mutation by radiation (Hollaender), and the production of penicillin-resistant strains of *Staph. aureus* (Demerec). The work discussed is concerned, for the most part, with methods of producing and selecting mutants of micro-organisms. A discussion by Hollaender considers the sensitivity of viruses and micro-organisms to radiation of various wave lengths.

Experiments described by Greenstein and Chalkley on the influence of nucleic acids on dehydrogenase systems are concerned with the most important but extremely complex relations of regulatory factors on the quantitative production of gene products. Interpretations of results are interesting but, as stated by the authors, they are speculative. Some quantitative control of enzyme production appears to be inherent in a given gene but certainly much of the control must be due to metabolic interrelationships as well as external environmental conditions. It is perhaps through these secondary effects that many single gene mutations appear to have multiple effects.

The publication includes discussions that followed presentation of several of the papers.

HERSCHEL K. MITCHELL, Stanford University, Cal.



# A Spectrophotometric Measurement of Adenosine Deamination \*

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Received April 2, 1946

## INTRODUCTION

The biological importance of adenine as a structural unit of nucleic acids and certain coenzymes, as well as its role in the transfer of phosphate, has been established, but most investigations have given relatively little attention to metabolism of the aglycone.

Studies by Parnas, Ostern and Mann (1934) and others (Conway and Cooke (1939); von Euler and Skarzynski (1940); and Kerr and Seraidarian (1945)) have demonstrated that most animal tissues catabolize phosphorylated adenosine derivatives first to adenosine and then, through deamination, to inosine. Muscle tissues offer a notable exception in that deamination takes place principally before complete dephosphorylation. This sequence of reactions occurs to a lesser extent in brain, blood and the auricle of the heart, but probably occurs to some extent in all tissues. The pathway of the reaction in a specific tissue is apparently dependent on the relative tissue-specific active concentrations of dephosphorylases, adenosine deaminase and adenylic acid deaminase. The existence of these latter two enzymes in muscle was demonstrated by Schmidt (1928, 1929) who described purification methods for both enzymes. Schmidt's nucleotide deaminase acted only on adenosine-5'-phosphate and not on adenosine-3'-phosphate. Using a highly purified preparation of Schmidt's enzyme, Kalckar (1944) demonstrated a relatively low activity on adenosine and none on adenosine-5'-di- and triphosphates. Enzymes acting specifically on adenosine-3'-phosphate and on adenine desoxyribose nucleosides and nucleotides have not been reported, although recent work by Greenstein and Chalkley (1945) has pointed out the existence of deaminating enzymes acting on intact ribose and desoxyribose nucleic acids. A series of papers by Duchateau-Bosson *et al.* (1940, 1942) have described investigations interpreted as having phylogenetic significance in the distribution of purine deaminases. The tissues of vertebrates were found to contain both adenosine and guanosine deaminases. In cold blooded vertebrates (salamanders)

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\* This work was supported by funds from the Nutrition Foundation.

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guanosine deaminase is replaced by guanine deaminase while in the invertebrates both of the nucleoside deaminases are replaced by enzymes acting on the uncombined purines adenine and guanine.

In most of the investigations mentioned, deamination has been determined by measurement of the ammonia released during the reaction. Borsook and Dubnoff (1940) reduced the method to a micro scale and used an enzyme preparation from the mold *Aspergillus wentii*. More recently Kalckar (1944) utilized a micro method based on changes of absorption spectrum taking place during the conversion of adenylic to inosinic acid. The procedure enables a nearly continuous measurement of the course of reaction and is especially suitable for kinetic studies.

For the present work this method has been further elaborated for application to investigations of the deaminases of *Aspergillus oryzae* and *Neurospora crassa*.

#### ABSORPTION SPECTRA FOR PURE COMPOUNDS

Although wave length values for maxima of absorption of various derivatives of adenine and hypoxanthine, as tabulated in the literature, show good agreement, the values for molecular extinction coefficients do not. Consequently, absorption spectra for several of the compounds have been redetermined under conditions to be used for deamination experiments. The compounds were made up in 1/15 *M* phosphate buffer at pH 7.0 in a concentration equivalent to 10  $\gamma$  of adenine/ml. Compounds were obtained from the following sources:

- Adenosine—A. D. McKay Co., N. Y.  
B. L. Lemke Co., N. Y.
- Adenosine-3'-phosphate—Schwartz Laboratories, N. Y.  
A. D. McKay Co., N. Y.
- Adenine—Eastman Kodak Co., N. Y.  
A. D. McKay Co., N. Y.
- Hypoxanthine—Hoffman-LaRoche, Inc., N. J.  
A. D. McKay Co., N. Y.

The same compounds from different sources give essentially the same absorption curves. Inosine was prepared from adenosine by enzymatic deamination. Preparation of the enzyme will be described subsequently. One ml. of enzyme solution (5 mg. dry weight) was added to a solution of 250 mg. of adenosine in 50 ml. of water. The mixture was covered with toluene and incubated for 9 hours. Released ammonia was neutralized with dilute hydrochloric acid at intervals. A determination of absorption spectrum indicated the reaction to be nearly complete in 4 hours. The solution was then evaporated to dryness and extracted twice with 10 ml. of boiling 80% ethanol. On evaporation of this extract the inosine crystallized. After one recrystallization from water the yield was 150 mg. This material was then recrystallized from 80% alcohol. Absorption curves did not change appreciably after the first recrystallization.

Elementary analysis of the final product gave C 44.43%, H 4.51%, N 20.67%. Calculated C 44.72%, H 4.47%, N 20.87%.

The absorption spectra determinations were made with a Beckman Spectrophotometer using 1 cm. quartz cells. Curves for five compounds are given in Fig. 1. Wave length is plotted against the density readings

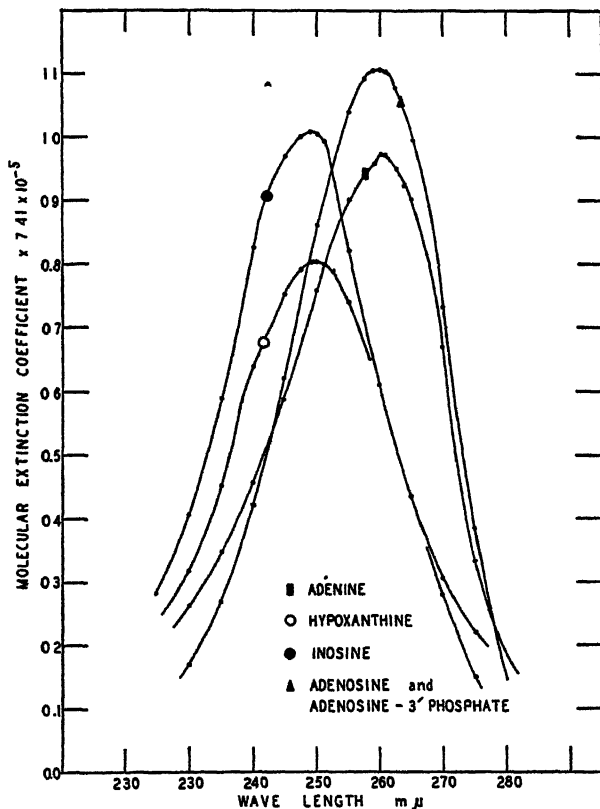


FIG. 1

Absorption Spectra of Adenine, Hypoxanthine, Inosine, Adenosine and Adenosine-3'-Phosphate in 1/15 *M* Phosphate Buffer at pH 7.0

taken directly from the spectrophotometric scale. With a 1 cm. cell  $\epsilon c = \log \frac{I_0}{I}$  = density reading where  $\epsilon$  = molecular extinction coefficient;  $c$  = concentration in mols/liter;  $I_0$  = intensity of incident light;

and  $I$  = intensity of emergent light. Maximum extinction coefficients under the experimental conditions described are given in Table I.

TABLE I  
*Maximum Molecular Extinction Coefficients*

Compound	Wave length $m\mu$	Max.
Adenine	260	$1.32 \times 10^4$
Hypoxanthine	249	$1.09 \times 10^4$
Adenosine	260	$1.49 \times 10^4$
Adenosine-3'-phosphate	260	$1.49 \times 10^4$
Inosine	249	$1.36 \times 10^4$

A determination of the spectrum of inosine-5'-phosphate isolated from muscle resulted in a curve essentially the same as that shown for inosine. The curve is not included, however, since the quantity of compound available would not permit an adequate determination of purity.

#### CALIBRATION CURVE

Kalckar (1944) has shown that a wave length of  $265 m\mu$  is particularly suitable for following the conversion of adenylic to inosinic acid. This is also true for adenosine. The curves of Fig. 2 indicate the absorption of adenosine and inosine as well as the calculated curves corresponding to 16 and 38% reaction. The series of black dots of  $265 m\mu$  represents experimental values for absorptions of various mol fractions of the two nucleosides. These points have been projected to give a calibration curve for the deamination in terms of *per cent* converted.

#### EXPERIMENTAL CURVES

Fig. 3 gives a series of experimental curves indicating total absorption changes during the course of enzymatic deamination of adenosine. The experiment was conducted as follows: A solution of enzyme containing 0.2 mg. of purified preparation per ml. was mixed with an equal volume of  $1/15 M$  phosphate (pH 7.0) containing 1.98 mg. of adenosine /ml. The mixture was sampled immediately and the sample diluted 1 to 100 in phosphate buffer. Additional samples were taken and diluted at 10, 20 and 120 minutes. The four samples correspond to the 0, 16, 38, and 90% conversion curves given in the Figure. The enzyme

preparation contributes a small amount of general absorption but it does not change during the reaction and may thus be subtracted from the readings of total absorption for utilization of the calibration curve.

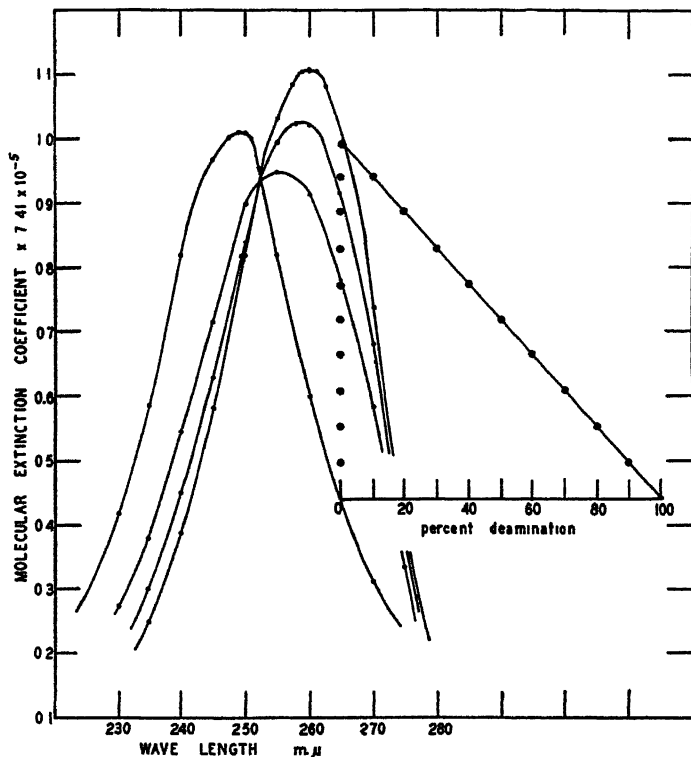


FIG. 2

Calibration Curve for Following the Enzymatic Deamination of Adenosine

The two extreme absorption curves represent those of inosine and adenosine while the two intermediate curves are calculated for 16 and 38% conversion of adenosine to inosine. Experimental points for absorption of mixtures of the two nucleosides at 265  $m\mu$  are projected to give the calibration curve in terms of *per cent* deamination.

## DISCUSSION

The absorption curves given in Fig. 1 were determined for conditions to be used in experimental work on studies of deamination. The considerable variation in molecular extinction coefficients found in the



literature may possibly be explained on the basis of solvent differences, pH differences or purity of the compounds. With respect to the former we have noted little change in using succinate rather than phosphate buffer. Relatively small changes in pH may contribute a great deal to variations in extinction coefficients, especially with hypoxanthine and

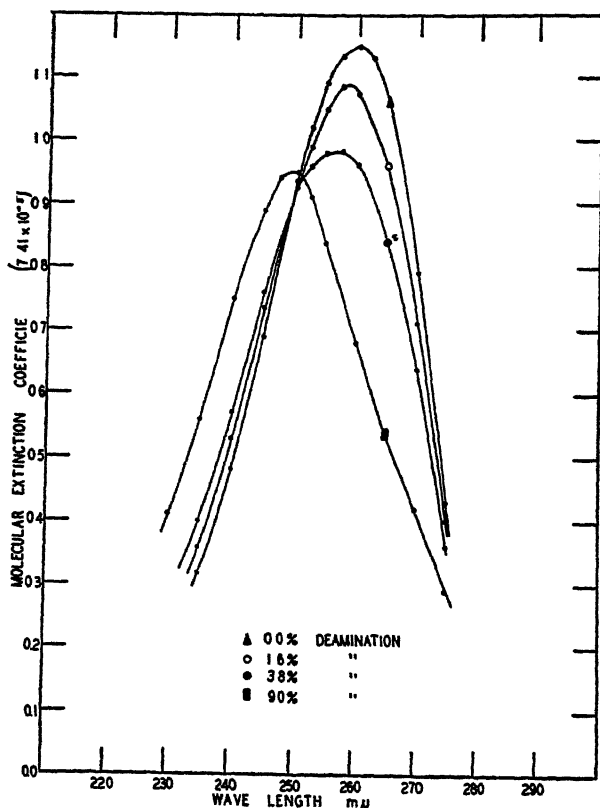


FIG. 3

The 4 Absorption Curves Represent 0, 16, 38 and 90% Deamination of Adenosine as Determined on Samples from a Reaction Mixture

its derivatives. Loofbourow and Stimson (1940) made a detailed investigation of the absorption changes of adenine with changing pH showing a shift in  $\epsilon$ -max from  $1.35 \times 10^4$  to  $1.08 \times 10^4$  with a pH change from 6 to 9. More recent work by Stimson and Reuter (1945)

indicates a less extreme change for adenosine. Investigations in this laboratory have demonstrated a considerable shift with pH change of both  $\epsilon$ -max and the wave length of  $\epsilon$ -max for the nucleoside inosine. Changes for hypoxanthine are even greater than those of inosine, adenine or adenosine.

The experimental and calculated absorption curves at various stages of reaction are in good agreement despite the small light-absorbing background due to the enzyme preparation. However, in a general application of this method of following the deamination reaction, using cruder enzyme preparations, this light absorbing background must be considered critically.

### SUMMARY

1. Ultraviolet absorption spectra are given for adenine, adenosine, adenosine-3'-phosphate, hypoxanthine and inosine. The determinations were carried out in phosphate buffer at pH 7.0 using a Beckman Spectrophotometer.

2. The spectrophotometric method of Kalckar (1944) has been elaborated for use in studies of adenosine deaminase in molds.

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# Adenosine Deaminase from *Aspergillus Oryzae* \*

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## INTRODUCTION

In 1914, Takamine described a preparation of a highly active diastase from the mold *Aspergillus oryzae*. The mold was grown on moist bran, dried and the enzymes extracted with water, followed by precipitation by alcohol. Dried preparations of an enzyme mixture produced in a similar fashion have been commercially available for many years under the names of takadiastase and clarase. The material is commonly labeled in terms of an arbitrary standard as percentage diastase activity, although the mixtures contain many enzymes. It has been found in this laboratory that takadiastase is an excellent source of an enzyme catalyzing the conversion of adenosine to inosine. This paper is concerned with further purification and some kinetic studies of this enzyme by means of a spectrophotometric method of following the deamination reaction (Kalckar, 1945, and Mitchell and McElroy, 1946).

## ENZYME SOURCE

Dried *Aspergillus oryzae* on bran and a number of takadiastase samples have been tested for adenosine deaminase activity. Results are given in Table I where the takadiastase samples are listed in terms of *per cent* diastase activity.

There is little correlation between the diastase and the deaminase activities but the samples with high diastase activity are more easily purified. The sample labeled 1230% was used for most of the experimental work described in this paper.

\* This work was supported by funds from the Nutrition Foundation.

† National Research Council Fellow (1945-'46). Present address, Department of Biology, Johns Hopkins University, Baltimore, Maryland.

TABLE I

*Adenosine Deaminase Activity of Takadiastase*

Activity is given in terms of  $\gamma$  of adenosine deaminated in ten minutes in a 1% solution of enzyme and a 0.198% solution of adenosine.

Diastase activity		Adenosine deaminated in 10 min. $\gamma$	Source
1.	<i>Aspergillus</i> bran	5	Dr. A. K. Balls, Western Regional Lab., Albany, California
2.	300%	40	Drugstore (Parke-Davis Co.)
3.	450%	50	Drugstore (Parke-Davis Co.)
4.	525%	160	Parke Davis Co., Michigan
5.	1230%	400	Parke Davis Co., Michigan
6.	1010%	140	Takamine Labs., N. J.
7.	1250%	70	Takamine Labs., N. J.
8.	1500%	110	Takamine Labs., N. J.

## GENERAL PROPERTIES OF THE DEAMINASE

Even the crude preparations of the enzyme are very soluble in water and a 25% solution can easily be prepared from purified material. The deaminase is precipitated by alcohol or acetone at room temperature without loss while dioxane causes some loss of activity. Sterile aqueous solutions may be preserved at 8°C. for many months and for at least a week at 25°C. Enzyme activity disappears to the extent of about 50% in 10 minutes at 80°C., although very little insoluble protein is produced. Ammonium and magnesium sulfates precipitate only part of the active enzyme even in saturated solution of the salts. The enzyme can be precipitated, however, by lead acetate and the activity recovered by  $H_2S$  or phosphate treatment. Dialysis against distilled water does not alter enzymatic activity in a 24-hour period. Preparations rapidly and permanently lose activity below pH 4 and above pH 9.5. The enzyme does not adsorb to an extent useful for purification on permutit, alumina, amberlite, calcium carbonate or charcoal (Norit) in the range of pH stability.

## ENZYME PURIFICATION

A number of variations in purification procedures have been utilized, but the simplest method giving consistent results is described as follows: One g. of takadiastase (1230%) was dissolved in 20 ml. of water and the solution centrifuged. The precipitate was discarded and the supernatant solution was shaken with 2 g. of permutit. After

centrifugation 2 g. of KCl were dissolved in the supernatant enzyme solution followed by 10 ml. of ethanol. The resulting precipitate was removed and discarded. Addition of 45 ml. more of ethanol precipitated essentially all of the adenosine diaminase. Following centrifugation, the precipitate was redissolved in 15 ml. of water and reprecipitated with 30 ml. of alcohol. The resulting precipitate (75 mgs. dry weight) was redissolved in 15 ml. of water and stored under toluene in the refrigerator. About 60% of the original activity was retained in the final preparation. This represents approximately an eight-fold purification over the takadiatase and about six hundred-fold purification over dry *Aspergillus oryzae* bran. Further purification by alcohol precipitation is inefficient and attempts at crystallization from water, acetone-water or alcohol-water were unsuccessful. The enzyme preparation as described was utilized in kinetic studies.

### ENZYME SATURATION

Determinations of the amount of adenosine required to saturate the enzyme were made in 1/15 *M* phosphate and in 0.2 *M* succinate buffers at pH 6.15. The enzyme preparation was diluted 1 to 10 with buffer, the pH was adjusted and the solution was then mixed with an equal volume of appropriately diluted adenosine solution. The reaction for the lowest concentration of adenosine (20  $\gamma$ /ml.) was carried out directly in the cell of a Beckman Spectrophotometer. Absorption changes at 265  $m\mu$  were recorded every minute. With higher substrate concentrations samples were removed at longer intervals and diluted in the appropriate buffer to give an equivalent of 10  $\gamma$  of adenine/ml. Since the quantity of substrate converted is essentially proportional to time up to about 20% reaction, several early readings were recorded and utilized to calculate the initial reaction velocity at each substrate dilution. The calculated rates ( $\gamma$  adenosine converted in 10 minutes) are plotted in Fig. 1. The experiments were carried out at 25°C. It may be observed that half saturation is reached at 50  $\gamma$  of adenosine/ml. in phosphate while 72  $\gamma$  are required in succinate.

Using a low concentration of substrate (20  $\gamma$ /ml.) and the 1 to 10 enzyme dilution, velocity constants at different time intervals have been calculated from the equation  $K = \frac{1}{t} \log \frac{a}{a-x}$  where  $K$  = velocity constant,  $t$  = time in minutes,  $a$  = initial concentration and  $x$  = quantity of substrate converted in time  $t$ . These values are listed in Table II.

The reaction appears to be pseudo-unimolecular up to 50% conversion.

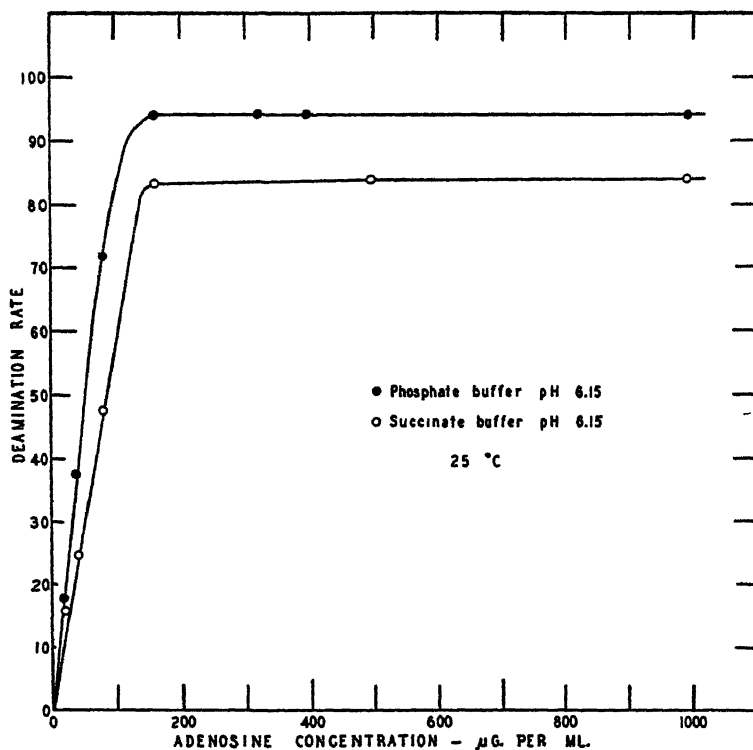


FIG. 1

The Quantity of Substrate Required to Saturate the Adenosine  
Deaminase Purified from Takadiastase  
The reaction mixtures contained 0.25 mg. of enzyme preparation/ml.

TABLE II

*Velocity Constants with the Enzyme Unsaturated*

<i>t</i> (Min.)	Per cent conversion	<i>K</i>
3	19	0.0183
4	24	0.0182
5	30	0.0176
6	37	0.0186
7	41	0.0186
8	44	0.0179
9	49	0.0177
10	52	0.0173
11	56	0.0173
12	59	0.0168

## PH OPTIMUM

Using the same general technique already described the pH optimum for the deaminase has been investigated. Experiments were carried out at the lower range of enzyme unsaturation (20  $\gamma$  of adenosine/ml.) in both phosphate and succinate buffers. A pH curve was also determined in phosphate buffer with substrate in excess of that required to saturate the enzyme. In all cases rate was determined as the quantity of adenosine converted in 10 minutes. As determined by a Beckman pH Meter, all pH changes during reactions were less than 0.1 unit. These data are summarized in Fig. 2.

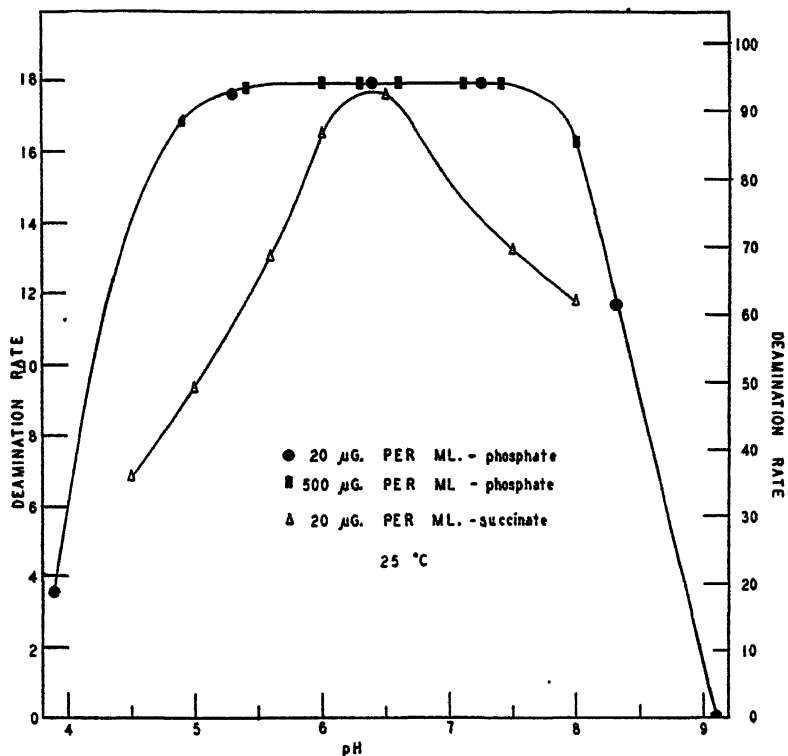


FIG. 2

The pH Optima of Adenosine Deaminase from Takadiastase as Determined in Different Buffers and for Two Substrate Concentrations

The deamination rate at the right corresponds to the high substrate concentration and the rate at the left corresponds to the low substrate concentration. The reaction mixtures contained 0.25 mg. dry weight of the enzyme preparation.



## TEMPERATURE OPTIMUM

The temperature optima of the adenosine deaminase were determined in phosphate buffer (pH 7.0) at substrate levels corresponding to partial enzyme saturation and above saturation. Temperatures were maintained in a thermostatically controlled water bath ( $\pm 0.1^\circ\text{C}$ ). Rates were determined as before and the resulting rate-temperature curves are given in Fig. 3.

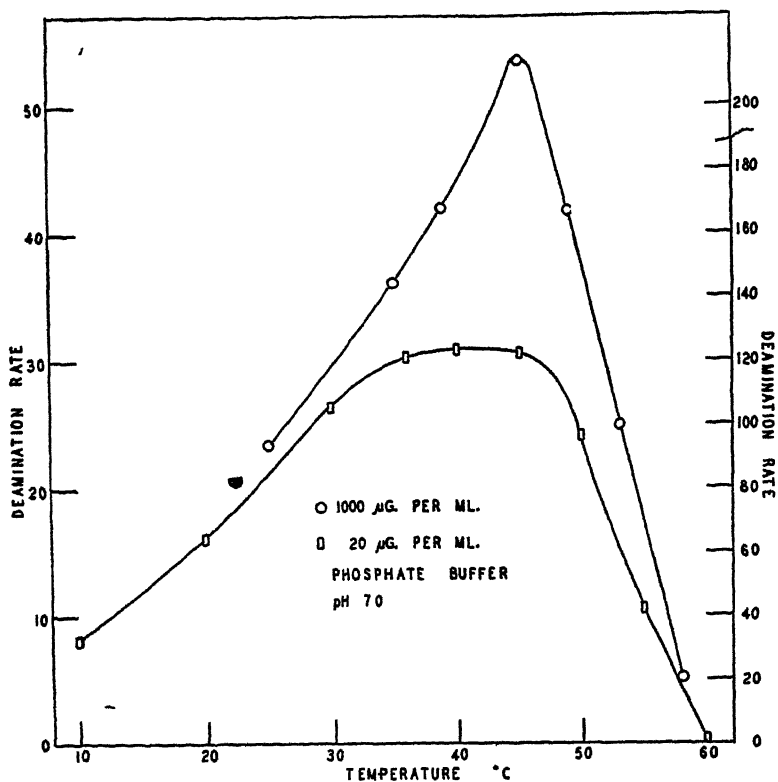


FIG. 3

## Temperature Optima of Adenosine Deaminase from Takadiastase

The deamination rate at the right refers to the high substrate concentration and the rate at the left refers to the low substrate concentration. The reaction mixture contained 0.25 mg. dry weight of the enzyme preparation.

Calculations of  $\mu$  (activation energy) and  $\Delta H$  (heat of denaturation) from the data for both substrates concentrations gave approximately 12,000 cal./mol for  $\mu$  and 75,000 cal./mol for  $\Delta H$ . The data are not sufficiently extensive for calculations of more than approximate values.

## DISCUSSION

Although the enzyme preparation described is considerably purified it still contains other enzymes than adenosine deaminase. In particular, dephosphorylases acting on adenosine-3'-phosphate and adenosine-5'-phosphate are still present. These compounds are thus deaminated by the enzyme preparation through the adenosine system. These reactions will be considered in a subsequent publication.

Data are insufficient for a rigorous comparison of the mold deaminase with a similar one from animal tissue, although the pH characteristics presented here are considerably different from those described by Conway and Cooke (1939). Solubility and stability to dialysis suggest that the enzyme from *Aspergillus* is a relatively simple protein without a dissociable prosthetic group. Striking stimulation by inorganic ions has not been noted. None of these facts are in serious discord with published information on the properties of adenosine deaminase from animal tissue.

Fig. 1 demonstrates a significant difference in dissociability of the deaminase-substrate complex as measured in phosphate and in succinate buffer. An apparent further manifestation of this phenomenon is strikingly illustrated in Fig. 2 where a broad pH optimum is shown in phosphate but not in succinate.

The lack of a sharp temperature optimum of the enzyme at low substrate concentrations may be interpreted as lack of protection of the enzyme by the substrate. Calculated heats of denaturation for the two substrate levels are not sufficiently accurate to verify this interpretation.

## SUMMARY

1. Takadiastase is shown to be a good source of adenosine deaminase. Purification of the enzyme is described.

2. Substrate combining capacity, pH optima and temperature optima have been determined under varied conditions using a purified enzyme preparation.

3. The deamination reaction is pseudo-unimolecular up to 50% conversion of adenosine to inosine.

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# The Amino Acid Composition of Cow and Human Milk Proteins

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## INTRODUCTION

The mixed proteins of mother's milk and cow's milk are generally recognized as being of the highest biological value. Although the relative status of cow's and mother's milk proteins is still undergoing investigation, the former impression, gained primarily from clinical studies, that human milk proteins are superior, is no longer held by some authorities (1, 2). In 1940, a comparative investigation was carried out on the essential amino acids in cow's and mother's milk. The results of these experiments were so similar to those found by Plimmer and Lowndes (3) in 1937 and to the then unpublished data of Beach *et al.* (4), that no detailed report was made. Early in 1944, we obtained a liter of milk from a white multipara, during her fourth month *post partum* (White A). The amino acids in the proteins obtained from this woman's milk were different from the values obtained previously, so that it was decided to reinvestigate the whole problem for individual variations.

## AMINO ACID ANALYSES

Proteins were prepared from all the samples of milk by precipitation with an excess of freshly prepared 50% trichloroacetic acid at 0°C. for 24 hours. The precipitate was washed thoroughly with cold 10%  $\text{CCl}_3\text{COOH}$  and the excess acid and lipids were removed by successive extraction with acetone, hot alcohol, hot benzene and ether. The white powdery protein was dried at 110°C. to constant weight and analyzed.

Nitrogen was determined by the Kjeldahl method; arginine, histidine and lysine were isolated by the silver precipitation method, duplicate determinations, (*cf* 5) or determined microbiologically, on two hydrolyzates at 10 different dilution levels

(6); tyrosine and tryptophan were determined by the Millon-Lugg method, four replicates (*cf* 5); phenylalanine was estimated by a modification of Kapeller-Adler's reaction, average of six determinations (*cf* 5); cystine and methionine were estimated by the Folin and Sullivan methods, respectively, average of four analyses (*cf* 5); and threonine was determined by the periodate *p*-hydroxydiphenyl method on four analyses (*cf* 5). Leucine, isoleucine and valine were approximated by the chromate-permanganate oxidation method (*cf* 5) in the 1940 experiments, and estimated by the microbiological procedure in the recent analyses (7, 8, 9) on two hydrolyzates at ten dilution levels.

### DISCUSSION

Our analytical data are summarized in Table I, while the results of Plimmer (3), Beach (4) and Williamson (10) are given in Table II. The results of all four groups of investigators are in good agreement, especially when one takes into consideration the differences in method of preparation of the proteins and the analytical techniques. There are, however, significant variations in arginine, tryptophan, cystine and other amino acids, sample White A being most unusual in this respect. This mother, the wife of a local physician, was 36 years old at the time of her second delivery and had nursed the previous baby (born in 1942) for nine months. She had an ample, almost excessive, supply of milk for both children, but in contrast to the other mothers, is not a professional donor. Unfortunately, by the time the analyses were started she had discontinued nursing and a second sample could not be obtained. She stated that both children appeared to do exceptionally well on the breast and are considered large for their age.<sup>1</sup> The other individual samples were obtained from one white (B) mother, age 32, with six children, and one colored woman, age 23, with two children. In each case, the milk was not collected until six months *post partum*. The pooled milk (1944) was from fifteen colored mothers at least three months *post partum*. The history of the 1940 milk samples was not recorded. The colostrum milk was obtained from six young mothers, sixteen to twenty-four years old, two to nine days after delivery, who were patients at the Flower and Fifth Avenue Hospitals. The cow's milk was the regular commercial product obtained locally.

In view of the large differences in cystine and tryptophan between the proteins of White A and the other mothers, these analyses were

<sup>1</sup> This woman has more than the usual quantity of body hair which is of masculine distribution. The excess hair is lost during pregnancy and returns *post partum*. She also states that she consumed a well-balanced diet during pregnancy and lactation, including the ingestion of at least one egg per day.

TABLE I  
*Amino Acids in Milk Proteins*  
 (Calculated to 16.0 g. of Nitrogen) <sup>2</sup>

Amino acid in g.	Cow's milk		Mother's milk					
	1940	1944	(spray dried) 1946	Pooled 1940	Pooled 1944	White A	White B	Colored
Arginine	4.3; 4.0	3.8; 3.2*	3.2±0.3*	5.0; 5.1	4.3; 3.8*	6.8; 6.7	3.7; 3.7	3.8; 3.6
Histidine	2.5;	2.6; 2.6*	2.6; 2.5*	2.7; 2.9	2.8; 2.6*	2.8; 2.6	2.7; 2.9	2.7
Lysine	7.5;	6.6; 7.2*	8.2±0.8*	7.2;	6.7; 7.2*	6.5; 5.6	6.7; 6.7	6.8; 6.6
Tyrosine	5.3±0.2	6.4; 6.4	6.2±0.1	5.1±0.1	5.2±0.1	5.1±0.1	5.4±0.1	5.4±0.3
Tryptophan	1.6±0.1	1.4; 1.4	1.7±0.2	1.9±0.1	1.6±0.2	3.3±0.1	1.7±0.1	1.5±0.1
Phenylalanine	5.7±0.3	5.7±0.4	6.5±0.3	5.9±0.3	5.3±0.4	3.1±0.2	5.5±0.3	5.8±0.3
Cystine	1.1±0.1	0.7; 0.7	0.8±0.1	3.4±0.1	1.6±0.2	4.0±0.2	2.1±0.1	1.4±0.1
Methionine	2.8±0.1	3.5; 3.7	3.4±0.1	2.0±0.3	2.2±0.1	2.5±0.1	2.4±0.1	2.3±0.2
Threonine	4.6±0.3	4.5; 4.5	4.7±0.1	4.6±0.3	4.5±0.1	6.4±0.3	4.5±0.1	1.8±0.1
Leucine	11.3±1.0**	10.6±1.1**	8.7±0.9*	(15)	9.8±1.0*	8.1±0.9*	10.2±1.1*	5.0±0.1
Isoleucine	6.2±0.8**	8.5±0.8*	7.2±0.8*	(5)	7.5±0.8*	5.5±0.6*	7.6±0.8*	7.9±0.8*
Valine	6.6±0.7**	8.4±0.9*	7.0±0.8*	(5)	8.3±0.8*	5.8±0.6*	9.9±1.0*	5.4±0.6*
								6.9±0.7*

\* Microbiological method.

\*\* The sample was reanalyzed in 1944 by the microbiological method.

○ Approximate only.

The recent communication of Vickery and Clarke (11) criticizing the method used by Block and Bolling of computing the amino acid content of proteins to a uniform content of 16% of nitrogen expresses the viewpoint of the protein chemist concerned solely with problems of protein structure. The viewpoint of the protein nutritionist, however, is entirely different because utilization of dietary proteins by animals can be studied best, at the present time, by the nitrogen balance sheet method. Hence, an amino acid analysis of a protein is most useful in protein nutrition as a chemical description of the nitrogen contained in it. However, because amino acid data calculated to 16% of nitrogen could be misconstrued, the values given in this paper are calculated in g. of amino acid/16.0 g. of nitrogen.

TABLE II  
*Amino Acids in Milk Proteins*  
 (Calculated to 16.0 g. of Nitrogen) \*

Amino acid in g.	Cow's milk proteins			Mother's milk proteins		
	Plimmer (3)	Beach (4)	Williamson (10)	Plimmer (3)	Beach (4)	Williamson (10)
Arginine	3.8	4.1	4.2	4.6	5.1	4.8
Histidine	1.7	1.9	2.0	1.6	1.5	1.8
Lysine	6.2	7.2	6.4	6.4	7.4	6.7
Tyrosine	5.3	6.4	5.5	4.7	6.2	5.2
Tryptophan	1.5	1.4	1.5	2.0	2.3	2.2
Phenylalanine			5.8			5.4
Cystine	1.1	0.7	0.9	3.2	2.5	2.9
Methionine	2.7	3.4	3.2	1.8	2.4	2.0
Threonine			4.9			4.5
Leucine			16.8			16.2
Isoleucine			5.4			5.3
Valine			5.4			4.7

repeated nine months later. In each case, the results agreed with the previous determinations. The results in Table I also suggested that there may be a difference in the cystine content of the proteins from white and colored women's milk. Therefore, further samples of milk were obtained from three white mothers and five colored mothers. The mean cystine values from the five white and six colored mothers was 2.67 and 1.49, respectively. Statistical analyses indicated that the results were significantly different even when White A was omitted from the calculations.<sup>2</sup>

As was to be expected, the amino acid pattern of colostral milk differed from that of mature milk. The data also suggest that there may be individual differences in the amino acid pattern of the total milk proteins from different mothers. The differences in the amino acid pattern of total milk proteins is probably a reflection of the variations in the casein-lactalbumin ratios. This variability is in con-

\* We are indebted to Professor Bernard Riess for the statistical analysis. He states that when the cystine contents of the proteins of the white and colored mothers' milks (omitting White A) were compared by the Fischer "t" test, the "t" value is 2.02 and the corresponding P value is between the 0.02 and 0.01 level. When the values for White A are included, the "t" value is 5.36 and the P value is beyond 0.001.

trast to the results of comparative studies on liver and muscle proteins which show that these proteins, even from widely different species have a relatively constant amino acid pattern (*cf* 5).<sup>4</sup>

#### ACKNOWLEDGMENT

We are indebted to Miss Helen Leighty of the Child Welfare Federation of New York City, for collecting the samples of mother's milk.

#### SUMMARY

1. Mother's milk and cow's milk proteins have been analyzed for nitrogen, arginine, histidine, lysine, tyrosine, tryptophan, phenylalanine, cystine, methionine, threonine, leucine, isoleucine and valine.

2. Pooled samples of mother's and cow's milk proteins differ markedly only in two amino acids—cystine and methionine, the essential acid being methionine. However, the total of the sulfur amino acids of both human milk and cow's milk proteins is approximately equal.

3. It would appear from a consideration of amino acid data only, that human milk proteins are *not* nutritionally superior to the proteins of cow's milk.

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<sup>4</sup> The paper of Hodson and Krueger (12) on the "Essential Amino Acid Content of Casein and Fresh and Processed Cow's Milk as Determined Microbiologically on Hydrolyzates" should be referred to in this connection.





# The Influence of Ascorbic Acid on the Anaerobic Respiration of Sweet Potato Slices

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In connection with a study of the influence of *L*-ascorbic acid on the aerobic respiration of the slices of sweet potatoes (*Ipomoea batatas* Poir), the influence of the acid on the anaerobic respiration of the slices was also tried. Briefly, the method consisted in cutting the roots into thin slices, washing the slices in running cold tap water for 15 to 24 hours and then determining the rates of CO<sub>2</sub> liberation in an atmosphere of nitrogen by means of a Warburg respirometer.

Following this procedure, it was found that the addition of ascorbic acid, either the *l*- or *d*- isomers, to the slices respiring anaerobically in an atmosphere of nitrogen caused the slices to stop giving off CO<sub>2</sub>. (Curves I and II in Fig. 1.) The ascorbic acid seems to have had a toxic action on the plant cells, for it was not possible to revive the aerobic respiration by replacing the nitrogen in the respirometer flasks by air. (Curves IA and IIA in Fig. 1.) Even after 5 hours, the aerobic respiration of the slices which had been treated with ascorbic acid anaerobically, was only about 25% of the control slices which had no ascorbic acid added.

Potassium cyanide caused some inhibition of the anaerobic CO<sub>2</sub> production of the sweet potato slices. When addition of cyanide was followed by addition of ascorbic acid to the slices, the cyanide prevented the ascorbic acid from inhibiting the anaerobic respiration. (Fig. 2.) Sulfhydryl compounds such as cysteine and glutathione, which showed practically no effect on the anaerobic respiration of the slices, like the cyanide, were found to prevent the added ascorbic acid from stopping the anaerobic respiration. The curves for cysteine are shown in Fig. 3.

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As previously mentioned, it was usually found necessary to wash the sweet potato slices for 15 to 24 hours in cold running tap water for the added ascorbic acid to exert its inhibitory action on the anaerobic carbon dioxide production of the slices. The necessity of washing the slices for this length of time suggests that possibly some

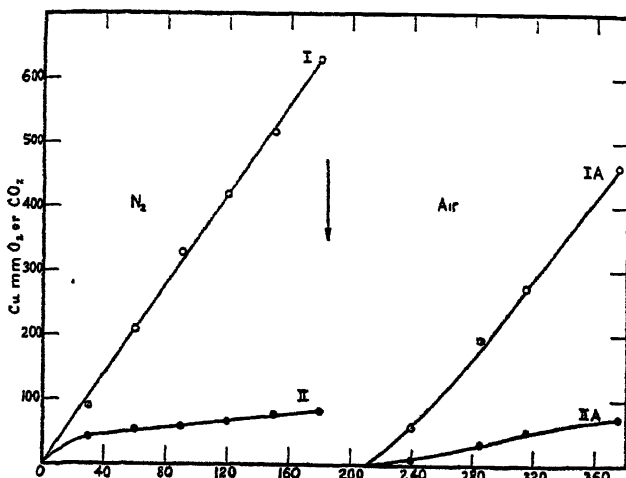


FIG. 1

Showing the inhibition of the anaerobic respiration of sweet potato slices by the addition of ascorbic acid, and also the inability of the slices to undergo aerobic respiration after replacing the nitrogen in the respirometer flasks by air. Thirty sweet potato slices (see experimental details), washed 18 hours in cold water, were placed in buffer solution in an atmosphere of nitrogen in the flasks. Curve I represents the rate of CO<sub>2</sub> given off by the slices in the absence of added ascorbic acid and curve II the rate of CO<sub>2</sub> given off in the presence of 2 mg. of added ascorbic acid.

At the end of 180 minutes the experiment was interrupted, the nitrogen was replaced by air, filter paper wicks moistened with 20% KOH were placed in the center wells of the flasks, and after the lapse of 25 minutes the rates of oxygen uptake were measured. Curve IA represents the oxygen uptake of the slices to which no ascorbic acid had been added, and curve IIA, the oxygen uptake of the slices to which 2 mg. ascorbic acid had been added.

substance was removed during the washing, the absence of which caused the slices to be vulnerable to the added ascorbic acid. Phosphate and magnesium ions are known to take part in anaerobic respiration. The absence of phosphates in the respiring slices was excluded, as

sodium potassium phosphate buffer was used. The addition of magnesium chloride also failed to prevent the inhibitory effect of the ascorbic acid.

Since acids, such as fumaric and citric, play a role in certain respiratory reactions, these acids were substituted for the ascorbic acid. Neither one, however, showed any tendency to interfere with the

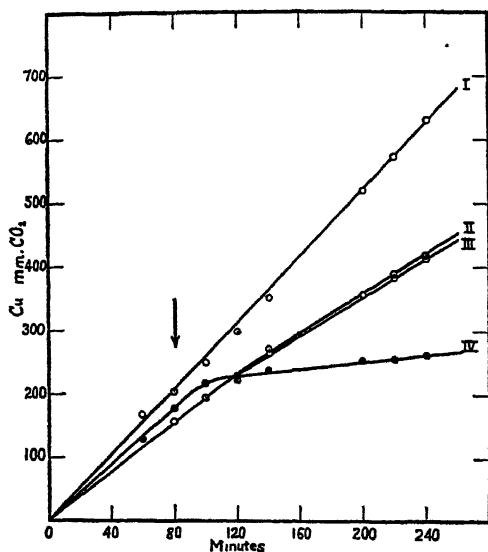


FIG. 2

Potassium cyanide preventing the inhibition of the anaerobic CO<sub>2</sub> production of sweet potato slices by added ascorbic acid. Thirty sweet potato slices, washed 20 hours in cold tap water, were placed in each respirometer flask together with a buffer solution in an atmosphere of nitrogen. (See experimental details). Curve I represents the rate of CO<sub>2</sub> given off by the slices respiring anaerobically in the buffer solution. Curve II represents the rate of CO<sub>2</sub> given off by the slices respiring in the presence of 5 mg. of KCN. Curve III is the rate of CO<sub>2</sub> given off by the slices in the presence of 5 mg. of KCN to which 3 mg. ascorbic acid were added from the side arm after the reaction had progressed for 80 minutes. Curve IV is the rate of CO<sub>2</sub> given off by the slices in the presence of 3 mg. ascorbic acid added after 80 minutes.

anaerobic CO<sub>2</sub> production of the slices. Fumaric acid was also tried in the presence of ascorbic acid but it had no effect in overcoming the inhibition caused by ascorbic acid.

To learn whether any change had occurred in the added ascorbic acid when it caused inhibition of anaerobic respiration, determinations

were made of the amounts of ascorbic acid in the solutions added, in the slices and in the liquid in the respirometer flasks. It will be noted in Table I that the ascorbic acid remained unchanged during the duration of the experiment.

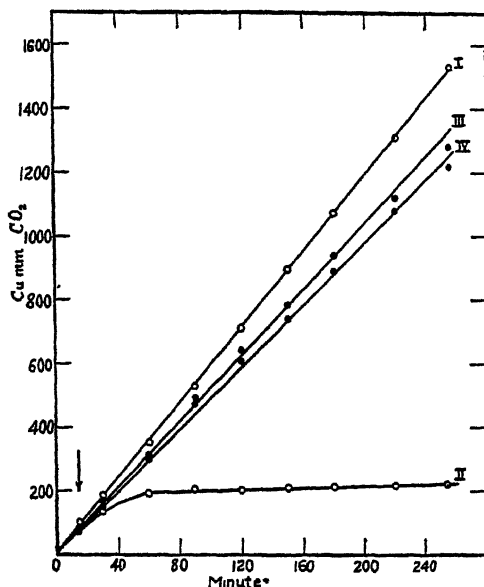


FIG. 3

The influence of cysteine on ascorbic acid inhibition of the anaerobic carbon dioxide production of sweet potato slices. Thirty-five slices, washed 18 hours in cold water, were used in each of the respirometer flasks together with a buffer solution. Curve I is the rate of CO<sub>2</sub> given off by the slices respiring in N<sub>2</sub> and buffer solution. Curve II is the same as I except 3 mg. of ascorbic acid were added after the experiment had been in progress for 15 minutes. Curve III shows the rate of CO<sub>2</sub> given off by the slices in the buffer solution containing 3 mg. cysteine to which 3 mg. ascorbic acid were added after 15 minutes. Curve IV shows the rate of CO<sub>2</sub> given off by the slices in the presence of 3 mg. cysteine.

Lots of 35 slices (washed in cold water 20 hours) were used in each of the respirometer flasks. Flask I also contained 2 ml. *M*/15 sodium potassium phosphate buffer solution and sufficient water to make the final volume of the reaction mixture 9 ml. and pH 5.2. Flask II was the same as I except that 1 ml. of a neutralized ascorbic acid solution and 1 ml. of a 30% metaphosphoric acid solution<sup>1</sup> were added from side

<sup>1</sup> The metaphosphoric acid was added to stop any possible oxidation of the ascorbic acid.

arms at the beginning of the experiment. Flask III was the same as II except that the 1 ml. of metaphosphoric acid was added at the end of the experiment.<sup>1</sup> The duration of the experiment was 240 minutes and the temperature, 25°C.

TABLE I

Dry weight of 35 slices	0.5892 g.		
Native ascorbic acid contained in 35 slices	0.53 mg.		
<i>l</i> -Ascorbic acid in 1 ml. of ascorbic acid soln.	3.03 mg.		
	Mg. ascorbic acid in flasks		
	I (control)	II	III
<i>At the beginning of experiment</i>			
In slices	0.53	0.53	0.53
In reaction soln.	0.0	3.03	3.03
Total ascorbic acid in flasks	0.53	3.56	3.56
<i>At the end of experiment (240 mins.)</i>			
In slices	0.51 <sup>2</sup>	0.29	0.30
In reaction soln.	0.00	2.87	2.87
Total ascorbic acid in flasks	0.51	3.16 <sup>2</sup>	3.17 <sup>2</sup>

Bearing in mind that the reaction in Flask II was stopped by metaphosphoric acid at the beginning of the experiment and in Flask III at the end of the experiment (240 mins.) it becomes evident that no change (beyond experimental error) occurred in the amount of ascorbic acid in Flask III during the duration of the experiment.

Ascorbic acid was also added to washed slices from other plants respiring in an atmosphere of nitrogen. Slices from beets, carrots and the common potato, *solanum tuberosum*, behaved like the sweet potato slices in that practically no carbon dioxide was given off. Slices from turnips, kohlrabi and a particular variety of sweet potatoes, Pelican Processor, failed to respond to the inhibiting action of the ascorbic acid. This failure of the ascorbic acid to prevent the evolution of CO<sub>2</sub> in the cases of the two last mentioned slices might possibly have been due to these slices not having been washed sufficiently in water. It might be mentioned that different varieties of sweet potatoes differed in the length of time the slices had to be washed in cold running tap water before their anaerobic CO<sub>2</sub> formation could be inhibited by the added ascorbic acid. Slices from some varieties appeared to require

<sup>2</sup> The loss in ascorbic acid in flasks II and III (3.56-3.16 and 3.17 mg.) was probably due to autoxidation in handling the ascorbic acid solution and to experimental error.

<sup>3</sup> The reason why less ascorbic acid remained in the slices in flasks II and III than in the slices in flask I was attributed to the eluting action of the metaphosphoric acid.

only a few hours of washing while others, those possessing highly pigmented tissue, required 20 or more hours of washing.

In the case of the beet slices, it was noticed that, when these slices failed to respire anaerobically in nitrogen, due to added ascorbic acid, the red pigment diffused into the reaction solution in the Warburg flask. This liberation of the pigment was not noticeable when the beet slices respired aerobically in the presence or absence of added ascorbic acid.

### EXPERIMENTAL

Sweet potatoes in a good firm condition with no signs of decay were used. The slices were prepared by first cutting off the two ends of the sweet potato, then a sharp cork borer was passed longitudinally through the tuber. This last operation was repeated as many times as the area of the surface permitted. The tuber was then cut horizontally by a microtome. In this way several hundred round discs or slices, 1.2 cm. in diameter and 400  $\mu$  thick were obtained from the same potato. The slices were placed immediately in running cold tap water and washed for 15 to 24 hours. During the washing the slices were continuously in motion and thereby thoroughly mixed. In this way it was found possible to obtain lots, consisting of 30-35 slices, which were shown to be very uniform or equivalent with respect to respiration measurements.

A Warburg respirometer was used for following the rate of carbon dioxide liberation in an atmosphere of nitrogen. The nitrogen was first passed over a heated coil of copper mesh to remove traces of oxygen present in it and then washed with water to saturate it with moisture at the temperature of the manometer bath, 25°C. While the respirometer flasks were shaking at the rate of 120 oscillations per minute, they were continuously flushed with the nitrogen during the 15 minute equilibrium period before starting the experiment. Oxygen uptakes were measured in air using filter paper wicks moistened with 0.2 ml. of 20% KOH in the center wells of the flasks for absorbing the carbon dioxide as it was formed. The capacity of the flasks was about 50 ml. Thirty to thirty-five slices were used in the flasks with 2 ml. of *M*/15 sodium potassium phosphate buffer at pH 5.2. Water was added to make the total volume up to 9.2 or 10.2 ml. including that of the slices and the various solutions added.

The solutions of ascorbic acid, glutathione, KCN, *etc.*, added to the respiring slices were prepared by dissolving the required amount in *M*/50 phosphate buffer and adjusting the pH to 5.2 by means of a few drops of an aqueous solution of alkali or acid.

The amounts of ascorbic acid in the slices and in the reaction solutions contained in the respirometer flasks were determined by a modification of the method described by Bessey and King (1) and King (2) using a visual titration with the sodium salt of 2,6-dichlorophenolindophenol in a 3% aqueous solution of metaphosphoric acid. In order to kill the slices and prevent oxidation of the ascorbic acid when the slices were removed from nitrogen to air, 30% metaphosphoric acid was added at least 40 minutes before the end of the experiment.

The authors wish to express their sincere appreciation to Mr. Stanley Lewis for his technical assistance in the work, and to the Rockefeller Foundation for financial aid.

## SUMMARY

*d*- and *l*-Ascorbic acid were found to inhibit the anaerobic respiration of washed sweet potato slices

Cyanide, glutathione and cysteine prevent the ascorbic acid from inhibiting the anaerobic respiration of washed sweet potato slices.

Fumaric and citric acids and magnesium chloride had no effect on the ascorbic acid inhibition of anaerobic respiration.

Carrots, beets and the common potato also showed inhibition of anaerobic respiration by ascorbic acid. Turnips, kohlrabi and the Pelican Processor variety of sweet potatoes apparently were not affected by ascorbic acid when respiring in nitrogen.

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# Reversal of Sodium Propionate Inhibition of *Escherichia coli* with $\beta$ -Alanine

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## INTRODUCTION

Many instances are known where by slight modification in structure a metabolite may be converted into an inhibitory compound (1). The similarity in structure of  $\beta$ -alanine and propionic acid suggested that the propionates might owe their bacteriostatic and fungistatic activity to a competitive interference with the synthesis or utilization of  $\beta$ -alanine or pantothenic acid. The following experiments were carried out to test this hypothesis. *Escherichia coli* was selected for the initial studies since it is capable of rapid growth in a synthetic medium free of  $\beta$ -alanine or pantothenic acid.

## EXPERIMENTAL

The medium used for the growth studies with *Escherichia coli* had the following composition:  $\text{Na}_2\text{SO}_4$ , 0.5%;  $\text{NH}_4\text{Cl}$ , 0.5%;  $\text{K}_2\text{HPO}_4$ , 0.8%;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.08%; asparagine, 0.6%; glucose, 0.5%; pH 7.35. Various amounts of the materials tested, adjusted to pH 7.35, were pipetted into bacteriological test tubes. All tubes of the test were then diluted to 5 ml. with water. Five ml. of the medium (double strength) were added and the test autoclaved at 12 pounds pressure for 10 minutes. After cooling to room temperature each tube received one drop of a 1:1000 dilution of a 24-hour culture of the organism that had been maintained by daily transfer in the medium described. Incubation was carried out at 37°C. until control tubes (no propionate or  $\beta$ -alanine) had reached maximal growth. Bacterial density was determined with the Klett-Summerson photoelectric colorimeter using a 540  $\mu$  filter.

The cultures assayed for pantothenic acid were autoclaved following growth and then were assayed directly for pantothenic acid. *Lactobacillus arabinosus* was used as the assay organism (2).

## RESULTS AND DISCUSSION

The data in Table I (Experiment 1) indicate that the bacteriostatic activity of sodium propionate for *Escherichia coli* may be counteracted

TABLE I  
*The Influence of Various Compounds on the Bacteriostatic Activity  
 of Sodium Propionate for Escherichia coli*

Expt. no.	Supplement Studied, per 10 ml.	Turbidity Readings Sodium Propionate, mg./10 ml.			
		0	10	20	50
1	None	85	24	10	5
	1 $\gamma$ $\beta$ -Alanine	85	70	29	12
	5 $\gamma$ $\beta$ -Alanine	85	84	70	33
	10 $\gamma$ $\beta$ -Alanine	85	85	71	37
2	None	80	32	13	10
	1 mg. $\beta$ -Alanine	80	61	47	21
	1 mg. Pantoyl lactone	75	33	15	10
	1 mg. Pantothenic acid	84	70	50	22
3	None	91	49	14	9
	2 $\gamma$ $\beta$ -Alanine	91	77	39	21
	2 $\gamma$ <i>dl</i> - $\alpha$ -Alanine	91	49	17	9
	2 $\gamma$ Thiamine chloride	98	59	29	12
	2 $\gamma$ Pyridoxine hydrochloride	90	49	25	10
	2 $\gamma$ Nicotinic acid	99	55	19	10
	2 $\gamma$ <i>dl</i> -Serine	91	41	14	9
	2 $\gamma$ Glycine	90	44	14	9
	2 $\gamma$ <i>dl</i> -Threonine	91	56	14	10
4	None	83	23	3	2
	2 $\gamma$ $\beta$ -Alanine	83	30	30	4
	2 $\gamma$ Inositol	83	27	3	2
	2 $\gamma$ Adenine sulfate	81	2	2	2
	2 $\gamma$ <i>p</i> -Aminobenzoic acid	88	49	8	2
	2 $\gamma$ Biotin	80	8	3	2
	2 $\gamma$ <i>L</i> -Tryptophan	88	26	3	2
	2 $\gamma$ <i>dl</i> -Valine	83	23	3	2
	2 $\gamma$ <i>L</i> -Glutamic acid	83	33	6	2
5	None	103	36	16	8
	0.1 $\gamma$ Adenine sulfate	101	21	14	7
	0.5 $\gamma$ Adenine sulfate	100	17	9	6
	2 $\gamma$ Adenine sulfate	104	8	6	5
6	None	101	30	8	7
	10 $\gamma$ Cytosine	104	89	12	7
	10 $\gamma$ Adenine sulfate	103	7	7	7
	10 $\gamma$ Uracil	103	20	7	7
	10 $\gamma$ Guanine	103	23	8	8
	10 $\gamma$ Uric acid	102	35	8	8
	10 $\gamma$ Allantoin	103	20	7	7

to a considerable degree by small amounts of  $\beta$ -alanine. Pantothenic acid similarly was effective but pantoyl lactone was inactive.

Several vitamins, amino acids and miscellaneous compounds were tested for their ability to antagonize the action of sodium propionate. Although some appeared to show a certain amount of activity, in those instances where it was observed, the compounds could be shown to stimulate the organism in the absence of sodium propionate. Separate experiments covering the entire growth period of *Escherichia coli* have shown that  $\beta$ -alanine in an amount sufficient to give the maximal obtainable reversal of the sodium propionate effect did not influence growth in the absence of sodium propionate.

Adenine sulfate and biotin were unique among the compounds tested in that they functioned synergistically with sodium propionate in inhibiting *Escherichia coli*.

The comparative bacteriostatic activity of sodium acetate, propionate and butyrate was studied. Sodium propionate was most effective in inhibiting the growth of *Escherichia coli* in the basal medium employed. In the presence of  $\beta$ -alanine, however, sodium propionate was no more toxic than its next higher or lower homologues (Fig. 1).

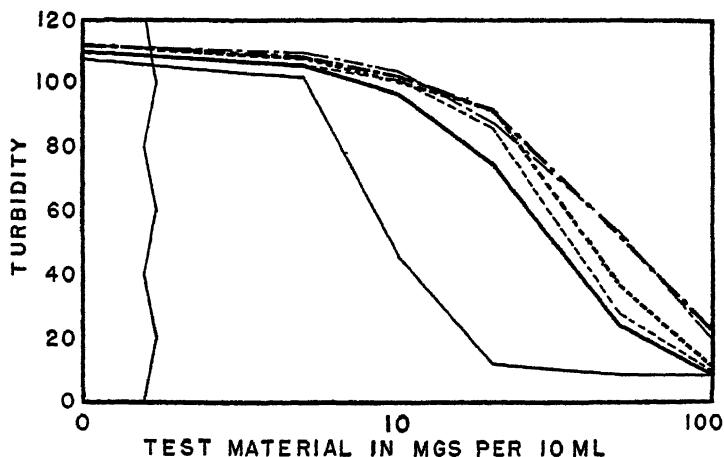


FIG. 1

The Influence of  $\beta$ -Alanine on the Bacteriostatic Activity of Sodium Propionate, Acetate or Butyrate for *Escherichia coli*.

Sodium propionate alone, — sodium propionate plus  $\beta$ -alanine, sodium acetate alone, ..... sodium acetate plus  $\beta$ -alanine, — · — sodium butyrate alone, — · · — sodium butyrate plus  $\beta$ -alanine.

Apparently sodium propionate inhibits two mechanisms in *Escherichia coli*, only one of which is concerned with  $\beta$ -alanine. For this reason the concentration of sodium propionate could not be varied widely and it was necessary to deal with amounts of the material that did not show the general inhibitory effect observed with fatty acids.

Further evidence in support of the thesis that sodium propionate owes its first inhibitory action on *Escherichia coli* in a purified medium to an interference with  $\beta$ -alanine synthesis is illustrated by the influence of  $\beta$ -alanine and pantoyl lactone on the synthesis of pantothenic acid by *Escherichia coli* in the medium described (Table II). While the

TABLE II  
*Pantothenic Acid Synthesis by Escherichia coli*

Substrate Added, per 20 ml.	Turbidity	Pantothenic Acid in $\gamma$ /ml.
None	155	0.10
1 mg. $\beta$ -Alanine	155	0.64
1 mg. Pantoyl lactone	154	0.16
1 mg. $\beta$ -Alanine and 1 mg. Pantoyl lactone	164	3.3

synthesis of pantothenic acid by *Escherichia coli* was stimulated in the presence of  $\beta$ -alanine and pantoyl lactone, or  $\beta$ -alanine alone, pantoyl lactone failed to influence significantly the synthesis of pantothenic acid. Thus, it appears that the limiting factor in the synthesis of pantothenic acid by *Escherichia coli* is the ability of the organism to produce  $\beta$ -alanine and not the synthesis of pantoyl lactone or the mechanism involved in coupling the two moieties.

The inhibitory effect of sodium propionate could not be reversed by  $\beta$ -alanine or increased by adenine with the following organisms: *Aspergillus clavatus*, *Bacillus subtilis*, *Pseudomonas sp.* and *Trichophyton mentagrophytes*. The reversal of sodium propionate inhibition  $\beta$ -alanine was, therefore, quite specific for *Escherichia coli*.

#### SUMMARY

The bacteriostatic action of sodium propionate for *Escherichia coli* could be antagonized significantly by small amounts of the structurally related metabolite,  $\beta$ -alanine.  $\beta$ -Alanine was specific, among the amino acids and growth factors studied, in showing this effect. Adenine, and to a lesser extent biotin, functioned synergistically with sodium propionate in inhibiting growth of *Escherichia coli*.

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# The Effect of the Level of Casein, Cystine and Methionine Intake on Riboflavin Retention and Protein Utilization by the Rat \*

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## INTRODUCTION

There is evidence to indicate that a relationship exists between protein metabolism and B-vitamin requirements. Hartwell (1, 2), Drummond *et al.* (3, 4) and Lecoq (5, 6) recognized as early as 1928 that rats fed increasing amounts of protein grew poorly unless their intake of B-vitamins was increased. Later work by Kleiber and Jukes (7) showed that riboflavin was involved in nitrogen utilization as the nitrogen content of chicks deficient in riboflavin was found to be less than that of pair-fed controls.

Sarett *et al.* (8, 9) observed that the urinary excretion of riboflavin in dogs and rats showed an inverse relationship to the level of protein intake and that the riboflavin content of the liver was increased from 17 to 30  $\gamma$ /g. when the level of protein in the diet was increased from 8 to 40%. Unna *et al.* (10) showed that the ingestion by rats of an 8% casein diet supplemented with 25 mg./day of *DL*-methionine tended to counteract the decrease of riboflavin in the liver caused by the low protein intake, although methionine supplementation did not increase the riboflavin content of the liver to that observed when 18% casein was fed. The addition of 25 mg./day of *L*-cystine was without effect, although it did result in slightly better growth. Czaczkes and Guggenheim (11) recently reported that the riboflavin content of the liver, kidney and muscle of rats was increased and that less riboflavin was excreted when the casein level of the diet was increased from 15 to 34%. These workers concluded, by analysis of the intestinal flora, that a greater synthesis of riboflavin occurred when rats were fed the low protein diet.

In the present work a more detailed study of the effect of the protein level in the diet upon the riboflavin content of the liver has been undertaken. The possible effects of cystine and methionine supplements with each level of protein on the growth, riboflavin content of the liver,

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riboflavin excretion, nitrogen balance, and the proximate composition of the liver have also been investigated to further clarify the interrelationships of riboflavin and protein metabolism.

### EXPERIMENTAL

In a preliminary experiment 18 weanling male rats (wt. 40–48 g.) of the Sprague-Dawley strain were fed diets containing 8, 18 and 50% casein, respectively, for 6 weeks. The rations were prepared at weekly intervals, fed *ad libitum* and stored in the refrigerator when not in use. The composition of these rations (B-8, B-18 and B-50) is shown in Table I. Two drops of a B vitamin supplement containing the following amounts of vitamins were fed daily: thiamine hydrochloride 25  $\gamma$ ; riboflavin 30  $\gamma$ ; niacin 25  $\gamma$ ; pyridoxine hydrochloride 25  $\gamma$ ; calcium pantothenate 200  $\gamma$  and choline 10 mg. This technique was used in order to keep the riboflavin intake constant. Two

TABLE I  
*Composition of Rations<sup>a</sup> and Food Consumption*

Ration <sup>b</sup>	Constituents, %						Average food consumption <sup>d</sup>
	Sucrose	Casein	dl-Methionine <sup>c</sup>		l-Cystine <sup>c</sup>		
			added	total	added	total	g /day
B-8	83	8	0	0	0	0.03	10.4
M-8	83	7.6	0.4	0.7	0	0.03	11.1
C-8	83	7.8	0	0.3	0.2	0.23	11.4
MC-8	83	7.4	0.4	0.7	0.2	0.23	11.2
B-18	73	18	0	0.6	0	0.06	14.3
M-18	73	18	0.4	1.0	0	0.06	14.7
C-18	73	18	0	0.6	0.2	0.26	15.2
MC-18	73	17	0.4	1.0	0.2	0.26	15.5
B-50	41	50	0	1.8	0	0.18	15.2
M-50	41	50	0.4	2.2	0	0.18	14.2
C-50	41	50	0	1.8	0.2	0.38	15.0
MC-50	41	49	0.4	2.2	0.2	0.38	14.4

<sup>a</sup> All diets contained 4% salts IV and 5% corn oil. Vitamins were fed by dropper as indicated in text.

<sup>b</sup> Designated B = basal, M = methionine, C = cystine; the number indicates the level of casein in the diet.

<sup>c</sup> Based upon utilization of both *d*- and *l*-methionine by the rat and literature values for methionine and cystine in casein as summarized by Block and Bolling (23).

<sup>d</sup> Each figure represents an average of the food consumption for the last 2 weeks of the experiment (6 rats per group).

drops of Haliver oil were given per week. These supplements were stored in a dark-colored bottle in the refrigerator when not in use.

Between the fourth and fifth weeks of the experiment, collections of the combined urine and fecal excretion were made in pyrex trays with sulfuric acid added as described by Schweigert *et al.* (12). The collections were homogenized in a Waring blender, made to a total volume of 500 cc., and stored under toluene in the refrigerator in dark-colored bottles prior to assay.

After withholding the vitamin supplement for 24 hours, the animals were decapitated and allowed to bleed thoroughly. The livers were removed and 1 g. samples

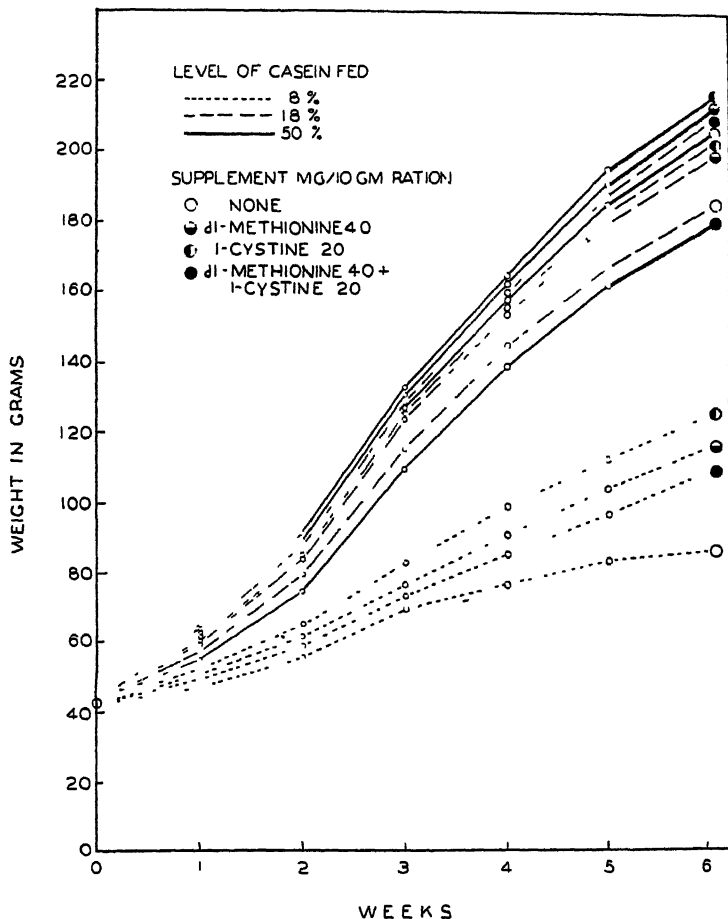


FIG. 1

Growth Curves Obtained for Rats Fed Diets Containing Varying Levels of Casein and Supplements of Methionine and Cystine



TABLE II  
*Effect of the Level of Protein in the Diet on the Riboflavin Content of the Liver  
 and the Riboflavin Excretion*

Diet	Riboflavin in liver $\gamma$ /g.			Riboflavin Excretion $\gamma$ /day		
	No. animals	Range	Ave.	No. animals	Range	Ave.
B-8	3	11.1-15.0	13.7	2	18.5-22.6	19.7
B-8	6	14.1-16.2	14.9	3	18.6-22.4	20.3
M-8	5	18.8-20.4	19.3	3	13.7-17.0	15.1
C-8	6	14.7-16.1	15.4	3	19.8-23.2	20.2
MC-8	6	17.8-21.4	19.0	3	14.6-17.0	16.4
B-18	3	22.7-22.7	22.7	2	14.3-14.3	14.3
B-18	6	21.5-26.8	23.2	3	15.3-16.6	15.9
M-18	6	20.4-29.3	26.2	3	13.5-14.9	13.8
C-18	6	21.1-28.0	25.1	3	15.3-15.9	15.7
MC-18	6	24.8-29.3	26.2	3	13.3-17.2	15.2
B-50	3	27.8-31.7	29.5	2	18.4-24.1	21.2
B-50	6	24.2-33.7	28.2	3	22.4-23.2	22.9
M-50	6	26.2-30.7	28.3	3	19.5-22.0	20.6
C-50	6	27.0-30.5	28.6	3	19.2-26.7	21.9
MC-50	6	21.4-25.4	23.8	3	20.0-21.8	21.0

TABLE III  
*Effect of the Casein, Methionine and Cystine Intake on the  
 Proximate Liver Composition \**

Diet	Per cent Nitrogen	Per cent Fat	Per cent Moisture
B-8	2.45	7.9	67
M-8	2.32	11.4	65
C-8	2.06	27.2	54
MC-8	2.33	9.9	66
B-18	3.00	1.0	70
M-18	3.14	2.2	69
C-18	2.92	2.8	70
MC-18	3.08	1.6	70
B-50	3.22	1.8	69
M-50	3.34	2.5	69
C-50	3.18	1.6	69
MC-50	3.39	1.9	69

\* Each figure represents the average of 2 determinations. Each determination was made on a composite of the livers from 3 rats as is indicated in the text.

were immediately weighed, homogenized with approximately 70 cc. of 0.1 N sulfuric acid and retained for riboflavin analysis. A fluorometric method, essentially that of Conner and Straub (13), with some modifications by Andrews (14, 15) was used for the riboflavin determinations. The results of the analyses are summarized in Table II.

Since a marked difference was found in the level of riboflavin in the livers of the animals which received the three levels of protein in the diet, 72 Sprague-Dawley weanling male rats (wt. 38–48 g.) were used in a second experiment with 6 rats in a group. Three of the diets fed were the same as the ones in the preceding experiment and served as controls. The effects of adding 40 mg. of *dl*-methionine and 20 mg. of *l*-cystine/10 g. ration on the riboflavin content of the liver were tested with each of the three diets which contained 8, 18 and 50% casein, respectively. The diets were designated with letters to indicate the amino acid supplement, and with numbers to indicate the level of protein fed: *e.g.*, B-8 for the 8% basal protein diet, M-18 for the 18% protein diet supplemented with methionine, C-50 for the 50% protein diet supplemented with cystine, *etc.* Vitamin supplements were fed as in the previous experiment. The composition of the rations and the food consumption for each group are summarized in Table I. The average rate of growth for each of the groups is presented in Fig. 1.

TABLE IV  
*The Effect of the Sulfur Amino Acids on the Nitrogen  
Utilization by the Rat\**

Diet	Average gain in weight g./day	Methionine intake <sup>b</sup> mg./day	Cystine intake <sup>b</sup> mg./day	N intake <sup>c</sup> N mg./day	Average excretion mg./day	Per cent nitrogen ingested in body gain <sup>d</sup>
B-8	1.0	29	3	117	66	42
M-8	1.7	74	3	125	50	60
C-8	1.9	31	26	129	52	60
MC-8	1.6	73	26	127	68	46
B-18	3.5	90	9	368	215	42
M-18	3.9	150	9	375	201	46
C-18	3.9	94	39	389	244	37
MC-18	3.7	157	39	397	199	50
B-50	3.9	266	27	1,080	909	16
M-50	4.1	305	27	1,010	798	21
C-50	4.1	262	57	1,060	819	24
MC-50	3.4	303	53	1,000	671	33

\* All data represent an average of the food consumption by 6 rats in each group, excretion data are an average obtained with 3 rats.

<sup>b</sup> See footnote (c) Table I.

<sup>c</sup> Calculated on the basis of 14.2% N in casein as obtained by the Kjeldahl method.

<sup>d</sup> (N intake—N excreted)/N intake  $\times$  100; the nitrogen excreted was determined by the Kjeldahl method.

Collections of total urinary and fecal excretions were made from 3 rats of each group. These collections were made between the fifth and sixth weeks of the experiment for the first six groups, and between the sixth and seventh weeks for the last six groups. The samples were stored in the refrigerator prior to riboflavin and nitrogen analyses.

Three rats of each group were sacrificed at the end of 6 weeks and the remaining rats at the end of 7 weeks. The livers were removed and suitable portions used for riboflavin analysis. The remaining portions of each of the 3 livers from each group of rats were pooled, dried at 105°C. and fat and nitrogen determinations made. The results of these analyses are shown in Tables II, III and IV.

### DISCUSSION

The rate of growth was increased when the 8% casein was supplemented with either cystine or methionine. However, when both were added somewhat slower growth was obtained than when the single amino acid supplement was fed (Fig. 1). Only a slight increase in the growth rate was noted when either of these amino acids was added to the 18% or 50% casein diet, although a retardation in growth was noted when both amino acids were supplemented to the latter level of casein. It should be pointed out that the methionine supplement with the 8% casein diet was adequate for normal growth requirements according to Rose (16). The increased rate of growth observed when the higher levels of protein were fed, therefore, indicates an insufficient supply of other amino acids or amino nitrogen *per se*. The slower rate of growth observed when both cystine and methionine were added to the 8% and 50% casein diets may be due to an imbalance of the ingested amino acids.

The levels of riboflavin and nitrogen found in the liver were similar to those reported by Sarett and Perlzweig (9), Unna *et al.* (10) and Schweigert *et al.* (17, 18) when similar levels of protein and riboflavin were fed. Although Sarett and Perlzweig did not obtain any differences in riboflavin retention when different levels of this vitamin were fed, Schweigert *et al.* (17) and Czaczkes and Guggenheim (14) observed that the riboflavin content of the livers was increased when additional amounts of the vitamin were fed. It should be pointed out that Sarett and Perlzweig varied all the B vitamins while the latter workers varied only the riboflavin intake.

The amount of riboflavin excreted was slightly less than the amount ingested when rats were fed 8% and 50% protein and the greatest excretion occurred when they were fed 18% protein. Excretion of riboflavin did not follow the exact inverse relation to the protein level of

the diet that was reported by Sarett and Perlzweig (8) and Czaczkes and Guggenheim (11). These differences may be due to the fact that starch was fed as a source of carbohydrate by the latter workers; in the present work sucrose was used as the carbohydrate. Earlier work showed that the type of carbohydrate influences the amount of riboflavin excreted (12, 19). It would be interesting to know whether the increased intestinal synthesis reported by Czaczkes and Guggenheim with low protein diets was due to the increased intake of starch as rice flour.

A progressive increase in the riboflavin content of the liver was observed when the protein level was increased. The greatest increase occurred when the protein level was increased from 8 to 18%. The supplementation of the diet containing 8% casein with methionine increased the riboflavin content of the livers from an average of 13.7 to 19.3  $\gamma$ /g. Although the ingestion of additional cystine resulted in an increased growth rate, it did not increase the riboflavin content of the liver. The addition of both methionine and cystine to the diet produced the same effect as methionine alone. These data show that the ingestion of methionine is necessary for the retention of riboflavin in the liver when rats are fed a low protein diet. The increased riboflavin retention that resulted when the high level of casein was fed was due, at least in part, to an increased level of methionine ingested, although other amino acids may have been involved. It is interesting to note that the amount of riboflavin in the livers was somewhat lower when animals received 50% casein plus both amino acids. The results obtained with methionine and cystine supplementation are essentially in agreement with those of Unna *et al.* (10), although the differences in the riboflavin values observed in the present work were greater and occurred in a shorter period of methionine supplementation. Also, since the methionine and cystine were added at the expense of the protein, the nitrogen content of each diet remained approximately the same and the differences produced could be ascribed more specifically to the amino acid added.

The nitrogen content of the livers was increased by feeding larger amounts of casein in the diet. This is in confirmation of the work of Harrison and Long (20). These workers also reported that the inclusion of 2% *DL*-methionine increased the amount of nitrogen in the liver. The smaller level of methionine added in the present experiment (0.4%) was without effect on the nitrogen content of the livers.

The effect of cystine, methionine and the level of protein on the production of fatty livers is shown in Table III. In support of the results of many workers (for bibliography see McHenry and Patterson (21)) it is clear that factors conducive to the production of fatty livers in these experiments were diets low in protein, particularly in methionine, and high in cystine. However, on an 8% protein diet the addition of methionine, the absence of additional cystine, and the inclusion of 10 mg./day of choline and only 5% fat in the ration were not sufficient to prevent fatty livers. Possibly the inclusion of inositol in the rations would have played a part in preventing fatty livers since Handler (22) showed that this is a lipotropic factor under certain conditions when a low protein diet is fed. The presence of high levels of fat in the livers did not affect the riboflavin content.

Supplementing the diets containing 8 or 18% casein with cystine or methionine had little effect on the total nitrogen excretion. However, when both were added to the 50% casein diet the excretion of nitrogen was considerably less. An increased efficiency of nitrogen utilization also appears evident with methionine or cystine supplementation of the 8% casein diets. The amount retained was not increased when both amino acids were added. However, the addition of these amino acids to the 50% protein diet resulted in an increased retention of nitrogen despite the fact that growth was retarded when this diet was fed.

Preliminary data on the excretion of methionine and the amount in the liver indicate that further metabolic changes occurred when the MC-8 and MC-50 diets were ingested. In general, an increased intake of protein and methionine caused an increased excretion of methionine, and a slight increase in the percentage of methionine in the liver protein. These effects will be further investigated.

We wish to express our thanks to Dr. D. K. O'Leary of E. I. Du Pont and Company, Wilmington, Delaware, for the *dl*-methionine used in these studies; to Merck and Company, Rahway, New Jersey, for supplies of the crystalline vitamins; and to Abbott Laboratories, North Chicago, for halibut liver oil.

#### SUMMARY

1. In confirmation of previous work, an increase in the amount of protein ingested increased the riboflavin retention in the liver of the rat. When diets containing 8, 18 and 50% casein were ingested, the amount of riboflavin in the liver increased from an average of 14 to 25 and 29  $\gamma$ /g. of liver, respectively.

2. About one-third of the increase in the riboflavin content caused by increased protein intake could be accounted for by the ingestion of increased amounts of methionine. None of the increase could be accounted for by the ingestion of increased amounts of cystine.

3. The growth of rats receiving 8% casein was increased with the additional intake of methionine or cystine. An increased intake of both methionine and cystine resulted in retarded growth when rats received 8 or 50% casein, but not with those receiving 18% casein.

4. The nitrogen content of the liver and the amount of nitrogen excreted were not appreciably influenced by methionine or cystine supplementation although both increased with high protein intake.

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# The Biochemistry of the Malaria Parasite \* VII. *In Vitro* Studies on the Distribution of Quinine between Blood Cells and Their Suspending Medium †

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## INTRODUCTION

The distribution of quinine between blood cells and plasma has been studied by several groups in the last few years. Grenfell, Richardson, and Hewitt (1) reported that, in infected (*P. lophurae*) ducks treated with quinine, the ratio of intracellular to extracellular quinine concentration is about 4 as compared to a ratio of about 1.5 in uninfected ducks. Similarly, Oldham *et al.* (2) administered quinine to normal and to infected (*P. gallinaceum*) chickens and found that the quinine concentration in the plasma and red cells was frequently higher in infected birds than in controls. These authors also pointed out that, whereas the ratio of the plasma to the erythrocytic quinine concentration was relatively constant for normal birds, the ratio in infected birds was much more variable.

*In vitro* studies on the distribution of quinine between erythrocytes and a synthetic medium were carried out by Silverman *et al.* (3). These investigators suspended washed blood cells from normal and infected

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(*P. gallinaceum*) chickens in an inorganic medium containing glucose. This simple suspending solution was substituted for plasma to reduce the number of variable factors present. It was found that the intracellular (I) extracellular (E) quinine distribution ratio of parasitized blood cells was generally higher and more variable than the ratio found with normal cells.

The purpose of the present investigation was to attempt determination of the reason for these differences in quinine distribution in parasitized blood cell samples as compared to normal samples. It was hoped that the information thus obtained could be related to the anti-malarial activity of the drug. The problem resolved itself into a study of the quinine distribution between blood cells and their suspending medium as a function of the following variables: parasite number, parasite type, parasitic surface area, intracellular water content, pH of the medium, composition of the medium and atmosphere, concentration of the various iron-containing blood pigments, reticulocyte count and the concentration of free protoporphyrin.

It was observed that the pH of the medium exerts an effect in that, as the pH increases, the I/E quinine ratio also increases. (It is generally known, however, that high pH values favor penetration of basic drugs into cells.) Evidence was also obtained showing that, with constant pH, the concentration of blood pigments within the cells influences the quinine distribution. Moreover, it was ascertained that the higher quinine concentrations and I/E ratios found with many parasitized cell samples, as compared to controls, do not especially require the presence of the parasites, as these effects can also be observed under circumstances in which parasites are absent. Our conclusions in this respect confirm those of Oldham *et al.* (2) who worked with anemic chickens. Finally, no direct correlation was found to exist between the quinine distribution and any one of the variables studied.

## MATERIALS AND METHODS

### *Source of Blood and Parasites*

Blood cells from normal, parasitized, or otherwise treated, adult white Leghorn chickens,\* chiefly cocks, weighing approximately 1 k. were used in these experiments. When parasitized blood cells were required, blood was obtained from birds infected

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\* The birds used in these experiments were supplied to us by Mr. William Cantrell of the Department of Bacteriology and Parasitology to whom we are grateful.

with *P. gallinaceum*, Strain 8-A, as designated by the Committee on the Terminology of Strains of Avian Malaria, of the American Society of Parasitologists. The infections were blood induced. Blood was drawn during the period of acute rise of infection on the third or fourth day after the infection of the bird or during the chronic period which follows the peak of infection.

#### *Preliminary Treatment of the Blood*

The saphenous vein of the chicken was pierced by a needle and blood smears were prepared for the study of the parasite distribution and the reticulocyte count. Blood was then withdrawn from the chicken by heart puncture with the aid of a 20 cc. syringe equipped with a No. 20 gauge needle and containing 1 cc. of a 10% solution of sodium citrate for each 10 cc. of blood to be drawn.

The citrated blood was then diluted with one volume of Krebs' calcium-free phosphate saline of pH 7.4-7.5 (4) and centrifuged at 1000 r.p.m. for five minutes. The sedimented cells were twice more resuspended in 50 cc. portions of the same phosphate-saline solution and centrifuged. The cells were finally resuspended in that volume of phosphate-saline solution which would give a red cell count of about  $2 \times 10^6$  cells/cu. mm. As previously reported (3), blood cells treated in this manner do not appear to suffer any apparent injury, as measured by their oxygen consumption as compared to that of the same cells, untreated and suspended in plasma.

#### *The Determination of the Quinine Distribution between the Red Cells and the Medium*

Unless otherwise specified, a control and a quinine-containing sample were prepared as follows for each experiment:

	Sample A	Sample B
Washed, oxygenated, blood cell suspension	2 volumes	2 volumes
1% glucose solution in phosphate-saline	1 volume	1 volume
Phosphate-saline solution	1 volume	—
$4 \times 10^{-6}$ M quinine in phosphate saline	—	1 volume

The concentration of quinine in sample B approaches that found in the blood of chickens treated with therapeutically effective doses of quinine (2).

The samples were mixed in Erlenmeyer flasks of such capacity that the gaseous phase was from one to two times that of the liquid phase.

The two samples were then shaken in a water bath at 30°C. for 15 minutes, unless otherwise specified. Following this incubation period (which was experimentally found to be of sufficient length to establish equilibrium) the vessels were removed from the bath and an aliquot of each sample was removed for a hematocrit determination. In the calculation of the cell volume and intracellular quinine concentration, the white blood cells were included with the red cells. Although the white cells normally take up considerably more quinine than do the red cells (5), it was found experimentally that essentially the same I/E quinine ratios were obtained whether the white cells were present or not. This was probably due to the fact that the number of white cells is relatively small compared with that of the red blood cells. Consequently, since it is difficult to separate white blood cells from parasitized red cells, the white cells were not removed in the experimental procedure employed.

The extracellular quinine concentration was determined by the analysis of the clear supernatant fluid from the hematocrit determination. The total quinine concentration of the cell suspensions was always  $10^{-6}$  M, but this was routinely checked by determining the quinine concentration of an aliquot of the sample after the incubation period. The intracellular quinine concentration was calculated by difference from the values for the extracellular and the total quinine concentrations of the sample. The validity of this procedure has previously been checked by direct assay of the centrifuged cells (3). It should be recognized that the values for intracellular quinine represent all the quinine taken from the medium by the blood cells, whether it be absorbed or adsorbed or both.

The fluorometric method of Brodie and Udenfriend (6) was employed for the estimation of quinine. Appropriate standard and blank solutions were prepared in addition to the experimental samples, and the comparisons were made in a Coleman photofluorometer with the aid of B<sub>1</sub>S and PC-1 filters. The concentrations of quinine are expressed in moles/l. of supernatant fluid and moles l. of red cells. The various other methods and procedures employed to study the factors under consideration in the different experiments will be discussed when reference is made to them in the course of this report.

## EXPERIMENTAL

### *Parasite Number, Parasite Type, Parasitic Mass and Quinine Distribution*

Differential parasite and reticulocyte counts<sup>\*</sup> were obtained from the blood smears which were prepared at the time of collection of the blood samples. The parasites were classified and the parasitic surface area was calculated by the method previously described (3).

From the results of representative experiments shown in Table I, it is clear that no direct relationship exists between any of these three factors—parasite number, parasite type and parasitic mass—and the distribution of quinine between the intracellular and extracellular phases.

However, in some of these and succeeding experiments it was observed that increased I/E quinine ratios were usually obtained with samples possessing increased reticulocyte numbers.

### *Intracellular Water Content and Quinine Distribution*

By vigorous centrifugation of the blood cell suspension after conclusion of the incubation period and removal of the last traces of supernatant fluid with blotting paper, it was possible to free the blood cell residue of supernatant fluid. The water content of the cells was determined by measuring the loss of weight of a portion of the blood cell residue after heating in a platinum crucible for about 16 hours at 101°C. The data were calculated on the assumption that the specific gravity of the cells was one.

The experimental data indicate that no direct correlation exists between the intracellular water content and the quinine distribution (Table II).

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<sup>\*</sup> We are indebted to Mrs. Jean Smith of the Department of Parasitology and Bacteriology of the University of Chicago for these measurements.

TABLE I

*Parasite Number, Parasite Type, Parasite Mass and Quinine Distribution*

Cells suspended in phosphate-saline, pH 7.4; 15 minute incubation at 39°C.

Parasites classified as: small, S; intermediate, I; large, L.

Type of infection: before peak, B; at peak, P; after peak, A; uninfected normal, N.

Exp. no.	Type of infection	Parasite distribution					Reticulo-cytes per cent	R.B.C./cc. of suspension $\times 10^9$	Parasitic surface area/R.B.C.	Quinine distribution		
		S per cent	I per cent	L per cent	R.B.C. infected per cent	No. of parasites /10,000 R.B.C.				I Intra-cellular concentration $M \times 10^{-3}$	E Extra-cellular concentration $M \times 10^{-4}$	I $\overline{E}$
									sq. micra			
1	P	7	90	3	95	19,652	2	1.04	24.2	6.28	2.28	28
2	P	3	96	1	82	15,680	<1	1.12	18.9	5.84	2.25	26
3	A	4	90	6	26	3,846	91	0.99	0.5	6.10	1.18	52
4	A	0	96	4	86	19,753	14	0.95	26.6	6.01	1.85	32
5	A	72	20	8	19	2,089	23	1.05	1.35	5.72	2.00	29
6	B	8	79	13	60	8,030	<1	1.09	12.7	4.91	2.53	20
7	B	54	43	3	46	8,474	<1	0.96	6.1	5.93	2.80	21
8	P	10	84	6	82	15,161	<1	1.23	19.8	3.88	1.64	24
9	N						<1	0.90		5.60	4.46	13
10	N						<1	1.04		5.09	3.55	14
11	N						<1	1.08		5.27	2.76	19
12	N						<1	1.07		4.64	3.60	13

*pH of the Medium and Quinine Distribution*

After the incubation period in each experiment, the pH of each sample was determined using a Coleman glass electrode. As shown in Table III and Fig. 1, the quinine distribution is greatly influenced by the pH of the medium. In general, as the pH increased from 7.0 and 8.0, the intracellular quinine concentration and the I/E quinine ratio increased. This is in accord with the generally accepted view that high pH values favor the penetration of weak bases into cells (7). Nevertheless, it is evident from the data of Table III that at the same pH, the intracellular quinine concentration and the I/E quinine ratio are usually higher for parasitized cells than for normal cells.

*Composition of Medium and Atmosphere and Quinine Distribution*

To ascertain what effects changes in the medium and gaseous phase might have on the quinine distribution, several experiments were performed in which Krebs' bicarbonate-saline solution (8) of the same pH was substituted for the usual phosphate-saline solution. In other experiments oxygen was replaced by nitrogen in the gaseous phase. These changes effected no significant differences in the quinine distribution observed (Table IV).

for cyanmethemoglobin. As seen in Fig. 2, these points are at or near peaks of absorption.

The blood cell samples were prepared for the spectrophotometric analysis as suggested by Dr. Dempsey B. Morrison (12). For the hemoglobin determination, 1 cc. or less of the blood cell suspension was diluted to 25 cc. with water and, after standing several minutes, the laked sample was centrifuged.\* Then 10 cc. of the clear super-

TABLE IV  
*Composition of Medium and Atmosphere and Quinine Distribution*  
15 minute incubation at 39°C.

Exp. no.	Type of blood sample	Suspending medium	Gaseous phase	Quinine distribution		
				I Intra- cellular concentration $M \times 10^{-6}$	E Extra- cellular concentration $M \times 10^{-6}$	$\frac{I}{E}$
1	Parasitized (66% infected R.B.C.)	Phosphate-saline, pH 7.4	Oxygen	3.65	1.35	27
			Nitrogen	3.54	1.35	26
2	Normal	Phosphate-saline, pH 7.4	Oxygen	3.46	3.15	11
			Nitrogen	3.43	2.98	12
3	Parasitized (80% infected R.B.C.)	Phosphate-saline, pH 7.5	Oxygen	7.10	2.93	24
		Bicarbonate-saline,	Oxygen	6.32	2.37	27

natant fluid were mixed with 0.5 cc. of 0.5% potassium ferricyanide and allowed to stand for 20 minutes. The solution was then mixed with 0.5 cc. of 0.1 *N* sodium cyanide and the resulting solution of cyanmethemoglobin was ready for analysis.

For the total pigment determination, 1 cc. or less of the suspension was added dropwise to about 22 cc. of acid acetone (2 cc. of conc. HCl/100 cc. of acetone) with constant agitation of the fluid. The mixture was diluted to 25 cc. with acid acetone and centrifuged in tightly corked tubes. The supernatant fluid contained the ferrihemic acid and was the solution used for the analysis.

The concentrations of both hemoglobin and the total iron-containing pigments were calculated for each wave length measured, by dividing the extinction value for the undiluted sample ( $\log I_0/I \times \text{dilution factor}$ ) by the extinction value for a con-

\* In later studies it was found advisable to add a cc. of 1 *M* phosphate buffer, pH 7.4, to the laked sample to stabilize the pH at 7.4. It was found that at pHs below 7.4, lower hemoglobin values were obtained because partial precipitation of the hemoglobin occurred as its isoelectric point, pH 6.8, was approached.

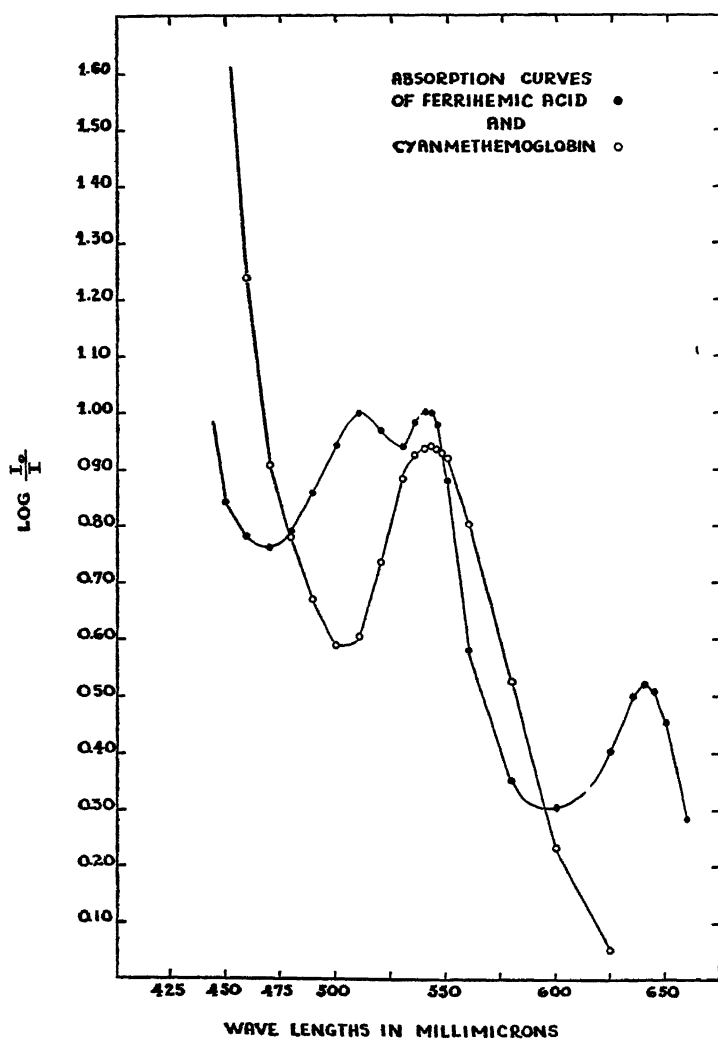


FIG. 2

## Absorption Curves of Ferrihemic Acid and Cyanmethemoglobin

Concentration of ferrihemic acid in acid acetone was  $9.2 \times 10^{-5} M$  while the concentration of cyanmethemoglobin in water was  $8.3 \times 10^{-5} M$ . The molarities are expressed in terms of the iron content. The normal blood cell suspension of Exp. 18, Table V, was used in the preparation of these solutions.

centration of 1 millimol/l. The concentrations are expressed in mM/l. where 1 mM indicates 1 millimol of porphyrin or iron, rather than 1 millimol of hemoglobin or ferrihemic acid.

$$c \text{ (concentration in mM/l.)} = \frac{E \text{ observed}}{\epsilon \text{ (} c = 1 \text{ mM/l.)}}$$

The  $\epsilon$  ( $c = 1$  mM/l.) values for cyanmethemoglobin were 11.5 at  $\lambda$  540, 11.5 at  $\lambda$  545, and 11.3 at  $\lambda$  548 m $\mu$ . The 11.3 value was calculated from some of our data on the basis of the other two values which were published by Drabkin (11). The  $\epsilon$  ( $c = 1$  mM/l.) values for ferrihemic acid were 11.3 at  $\lambda$  510, 11.3 at  $\lambda$  540, and 5.8 at  $\lambda$  640 m $\mu$ . These values were obtained by the spectrophotometric analysis of six samples of normal blood cell suspensions for hemoglobin and total pigment. First the concentration of hemoglobin in each sample was determined by using the  $\epsilon$  ( $c = 1$  mM/l.) values for cyanmethemoglobin. Then, with the assumption that the conversion of hemoglobin into ferrihemic acid in acid acetone is quantitative, the  $\epsilon$  ( $c = 1$  mM/l.) values for ferrihemic acid were calculated from the  $E$  observed values.

Hemoglobin has been found to be the only soluble pigment present in detectible amounts in laked parasitized (*P. knowlesi*) blood (10). It was assumed that a similar situation existed in chickens infected with *P. gallinaceum*. Consequently, the presence of any parasitic pigment (ferrihemic acid) in the blood cell suspensions used in these experiments did not complicate the spectrophotometric determination of hemoglobin.

The difference between the values for total iron-containing pigments and hemoglobin and its derivatives represented the value for the free ferrihemic acid present in the cells.

These experiments indicate that no direct relationship exists between the quinine distribution and either the free ferrihemic acid or hemoglobin concentration (Table V). However, there is some correlation between the total iron-containing pigments and the intracellular quinine concentration as shown in the calculation of the ratio of intracellular quinine concentration to the total pigment concentration. This ratio was found to be lower for normal cells than for parasitized cells. Since the variations in the intracellular total pigment concentration were observed to be relatively small, it seemed that the relative constancy of the above mentioned ratio might have been more apparent than real. The hemoglobin and total pigment concentrations in our system could be varied by changing the concentration of red cells present. Such a study has already been reported by Silverman *et al.* (3) who showed that, with a constant amount of quinine, as the red cell concentration increased, both the intracellular and extracellular quinine concentrations decreased while the I/E quinine ratio remained relatively constant. The changes in the quinine concentrations, however, were not directly proportional to the changes of the red cell concentration. To test whether the presence of ferrihemic acid and hemoglobin does influence the quinine distribution, the following experiments were performed. Crystalline hemin dissolved in phosphate-saline on the one hand, and hemoglobin in the form of a solution of laked blood cells in phosphate-saline on the other, were added in varying amounts to the normal red cell suspensions. It was found (Table VI) that as the extracellular hemin concentration increased from 0 to  $1.5 \times 10^{-4} M$ , the I/E quinine ratio decreased from 15.4 to 7.6. The extracellular concentrations of hemin employed in these experiments were smaller than the intracellular concentrations of malarial

TABLE V  
*Concentration of Iron-Containing Pigments and Quinine Distribution*  
 Cells suspended in phosphate saline, pH 7.4; 15 minute incubation at 39°C.  
 In every case there were  $1.0 \times 10^8$  R.B.C./cc. of suspension.

Exp. no.	Type of infection	Quinine distribution			I E	Exp. no.	(a) Intracellular concentration of iron pigments $M \times 10^{-3}$	(b) Intracellular concentration of hemoglobin $M \times 10^{-3}$	(a-b) Free farnemic acid $M \times 10^{-3}$	$\frac{I}{(a)}$ $\times 10^{-3}$	$\frac{I}{(b)}$ $\times 10^{-3}$	$\frac{I}{(a-b)}$ $\times 10^{-4}$	$\frac{I-3.2(b)-10^{-18}}{(a-b)} \times 10^{-3}$
		I Intracellular concentration $M \times 10^{-3}$	E Extracellular concentration $M \times 10^{-6}$										
1	A (3%)	5.46	1.80		30	1	11.1	11.2	—	4.93	4.88	—	—
2	A (0.1%)	4.98	2.13		23	2	12.4	12.8	—	4.01	3.90	—	—
3	B (58%)	5.65	2.10		27	3	13.8	10.7	3.1	4.09	5.29	18	7.2
4	B (79%)	5.80	2.50		23	4	14.1	12.7	1.4	4.11	4.56	41	12.4
5	P (88%)	5.32	2.23		24	5	14.2	13.2	1.0	3.75	4.03	53	11.0
6	B (78%)	4.87	2.28		22	6	12.1	10.6	1.5	4.02	4.59	33	9.8
7	A (27%)	6.05	1.85		33	7	10.4	9.3	1.1	5.81	6.50	53	28.0
8	A (19%)	5.72	2.00		29	8	13.4	13.4	—	4.27	4.27	—	—
9	B (59%)	5.50	2.53		22	9	14.6	13.4	1.2	3.77	4.11	46	10.1
10	B (60%)	4.91	2.53		19	10	15.0	14.4	0.6	3.27	3.41	82	5.0
11	B (46%)	5.93	2.80		21	11	14.8	14.8	—	4.00	4.00	—	—
12	B (48%)	4.84	2.15		23	12	14.1	13.0	1.1	3.43	3.72	44	6.2
13	P (91%)	4.74	1.51		31	13	14.0	12.9	1.1	3.38	3.67	43	5.5
14	P (82%)	3.88	1.64		24	14	12.6	10.5	2.1	3.08	3.69	19	2.5
15	A (45%)	5.37	1.75		31	15	10.6	8.3	2.3	5.07	6.48	23	11.8
16	N	5.09	3.55		14	16	16.9	16.9	—	3.00	3.00	—	—
17	N	5.27	2.76		19	17	15.5	15.5	—	3.39	3.39	—	—
18	N	4.64	3.60		13	18	15.3	15.3	—	3.03	3.03	—	—
19	N	5.01	4.28		12	19	15.7	15.7	—	3.19	3.19	—	—
20	N	4.71	4.59		10	20	14.3	14.3	—	3.30	3.30	—	—

\* The average  $\frac{I}{I_0}$  value of the 5 normal samples (Exp. no. 16-20) was  $3.2 \times 10^{-3}$ .



pigment (terrihemic acid) found in many parasitized blood samples. When larger concentrations of hemin were used, a considerable portion of it would precipitate under our experimental conditions. The addition of the hemoglobin solution to the red cell suspension had a similar effect to that of hemin (Table VII). As the extracellular hemoglobin concentration increased from 0 to  $2.7 \times 10^{-4}$  M to  $5.4 \times 10^{-4}$  M, the I/E quinine ratio changed from 15 to 12 to 10. The intracellular hemoglobin concentration in the blood cell sample used was about  $13 \times 10^{-3}$  M. Whether hemoglobin bound in the red cells and terrihemic acid present as malarial pigment in parasitized red cells behave in the same fashion as they did dissolved in the extracellular phase cannot be concluded from these experiments.

TABLE VI

*Effect of Added Hemin on Quinine Distribution*

Normal blood cells suspended in phosphate saline, pH 7.4; 15 minute incubation at 39°C.

Experiment 1: A—no hemin solution added; B, C and D received 1 cc., 2 cc. and 4 cc., respectively, of a saturated hemin solution. In each case there was  $8.26 \times 10^9$  R.B.C. suspended in a final total volume of 8 cc. The hemin was dissolved in phosphate-saline at pH 8.4.

Experiment 2: A—no hemin suspension added; B and C received 2 cc. and 4 cc., respectively, of hemin suspension. In each case there was  $7.32 \times 10^9$  R.B.C. suspension contained  $4 \times 10^{-3}$  M hemin in phosphate-saline at pH 7.6.

Exp no.	Intracellular concentration of total pigments $M \times 10^{-3}$	H Extracellular concentration of total pigments $M \times 10^{-3}$	Quinine distribution			$\frac{C}{H} \times 10^{-2}$	$\frac{E-control}{H-control} \frac{E}{H} \times 10^{-2}$
			I Intracellular concentration $M \times 10^{-3}$	E Extracellular concentration $M \times 10^{-3}$	$\frac{I}{E}$		
1A	12.7	0.55	5.22	3.40	15.4	6.2	—
1B	13.2	0.61	5.20	3.54	14.7	5.8	2.3
1C	13.1	1.02	4.61	4.31	10.7	4.2	1.9
1D	13.3	1.55	3.88	5.14	7.6	3.3	1.7
2A	13.7	0.22	5.75	3.80	15.1	17.2	—
2B	11.7	8.65	2.75	6.99	3.9	0.8	0.4
2C	14.2	14.1	2.74	6.76	4.1	0.5	0.2

*Reticulocyte Counts, Concentration of Free Protoporphyrin and the Quinine Distribution*

Since, as previously mentioned, high I/E quinine ratios were found in parasitized blood samples possessing high reticulocyte counts and since the intracellular quinine total pigment ratio was observed to be greater for parasitized than for normal blood samples, it was decided to study the quinine distribution in the blood from normal chickens treated with phenylhydrazine to increase their reticulocyte counts. The purpose of

this study was to determine whether uninfected blood cell samples with high reticulocyte numbers showed  $\frac{\text{intracellular quinine}}{\text{total pigment}}$  ratios comparable to those found with parasitized samples.

The phenylhydrazine was injected intravenously in the form of 1% solution of the hydrochloride in sterile saline. The dosage administered was 30 mg. of phenylhydrazine hydrochloride/kg. body weight as recommended by Wright and Van Alstyne (13). Usually 2 or 3 days were allowed to elapse after the injection before the next sample of blood was withdrawn. The results of these studies are shown in Fig. 3.

TABLE VII

*Effect of Added Hemoglobin on Quinine Distribution*

Normal blood cells suspended in phosphate saline, pH 7.4; 15 minute incubation at 39°C.

A.—no hemoglobin solution added; B and C received 2 cc. and 4 cc., respectively, of hemoglobin solution. In each case there were  $8.26 \times 10^9$  R.B.C. suspended in a final total volume of 8 cc. The hemoglobin solution consisted of laked R.B.C. in phosphate-saline; 1 cc. of this solution was equivalent to  $0.62 \times 10^9$  R.B.C.

	Intracellular concentration of hemoglobin $\times 10^{-3}$	G Extracellular concentration of hemoglobin $\times 10^{-4}$	Quinine distribution			E G $\times 10^{-2}$	E-control E G-control G $\times 10^{-2}$
			I Intracellular concentration $M \times 10^{-2}$	E Extracellular concentration $M \times 10^{-2}$	I E		
A	14.3	0.12	6.50	4.31	15.0	36.0	—
B	13.0	2.73	5.70	4.64	12.3	1.70	0.12
C	11.8	5.35	5.17	5.03	10.3	0.94	0.14

\* Expressed in terms of moles of iron.

\* The differences in intracellular concentration of hemoglobin can be explained on the basis of cell volume. The cell volumes of B and C were found to be 9% and 17%, respectively, greater than the cell volume of A.

In this series of experiments, the free protoporphyrin content of the cells was determined by the method of Grinstein and Watson (14) modified in two respects: (1) As suggested by Dr. Samuel Schwartz, the protoporphyrin was extracted from the cells by grinding them with the solvent in a fritted glass funnel with the aid of a pestle, and (2) the absorption of the protoporphyrin solutions was measured in a Beckman spectrophotometer. Since the absorption spectrum of protoporphyrin in 5% hydrochloric acid was found to have a strong peak at a wave length of 406–407  $m\mu$  and a much weaker one at 555  $m\mu$  (Fig. 4), the spectrophotometric readings were made at the former wave length. A standard curve for the absorption of protoporphyrin at a wave length of 406  $m\mu$  was obtained by using various dilutions of a hydrolyzed sample of the crystalline methyl ester of protoporphyrin (Fig. 5). This curve was used in the determination of the protoporphyrin concentrations. The crystalline methyl ester of protoporphyrin was kindly supplied by Dr. Samuel Schwartz.

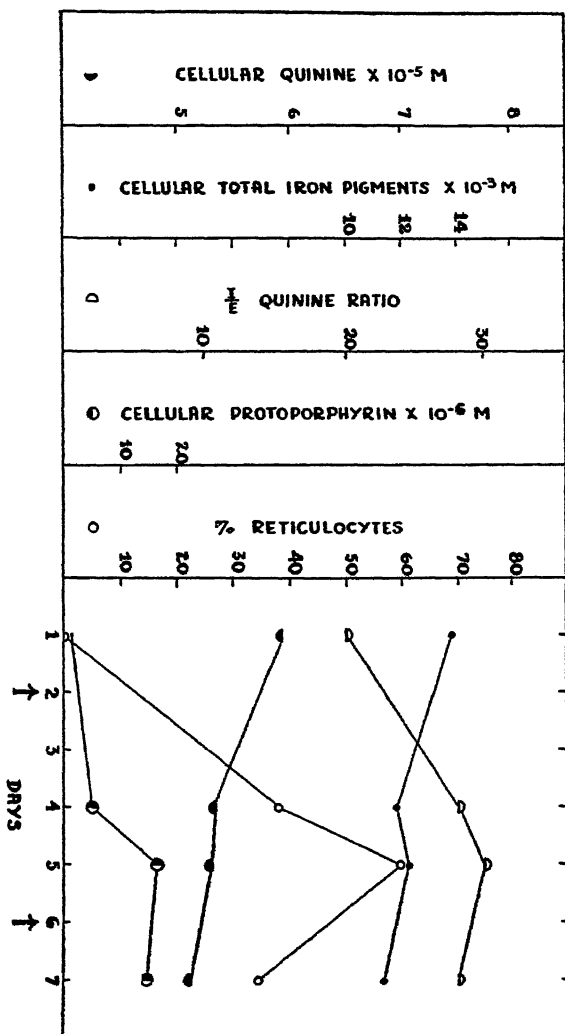


FIG. 3

The Effect of Intravenous Injection of Phenylhydrazine into a Normal Chicken upon the Reticulocyte Number, the I/E Quinine Ratio and the Cellular Concentration of Protoporphyrin, Total Iron-Containing Pigments and Quinine

The arrows indicate the days on which 30 mg. of phenylhydrazine hydrochloride (1% in saline)/kg. body weight were injected. The bird died on the eighth day following a third injection of phenylhydrazine.

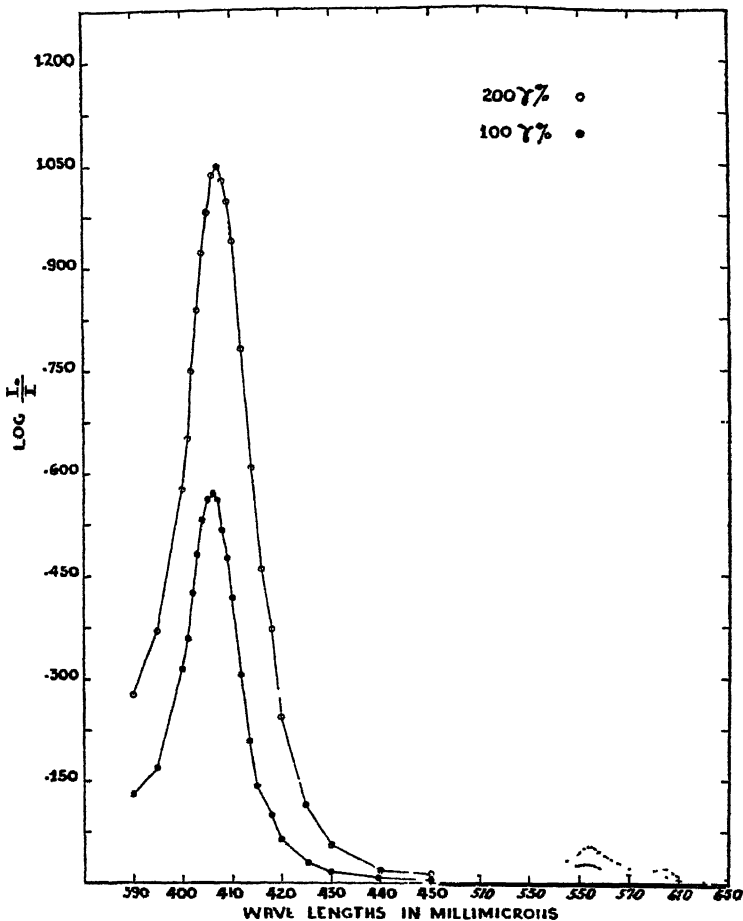


FIG. 4

## Absorption Curves of Protoporphyrin

The protoporphyrin was obtained by hydrolyzing its crystalline dimethyl ester for 16 hours at 5°C. with 25% HCl. The spectrophotometric determinations were made in 5% HCl.

As shown in Fig. 5, the changes which occurred in the I/E quinine ratio, following the administration of the phenylhydrazine to the chickens, paralleled more closely the changes in cellular protoporphyrin concentration than they did those in reticulocyte numbers or cellular iron pigment concentration.

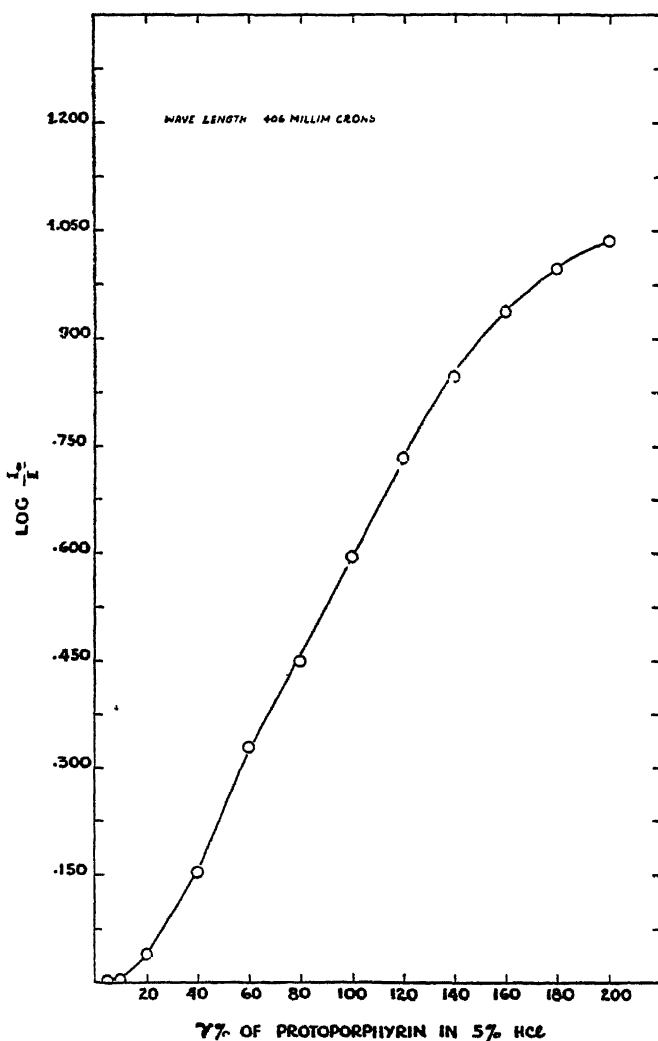


FIG. 5  
Standard Absorption Curve of Protoporphyrin

As Seggel (15) originally reported and as Watson and his coworkers (16) have recently confirmed, the protoporphyrin in the blood stream apparently exists in the "fluorescytes." These immature red cells, which give a red fluorescence in ultra-

violet light, appear in the blood stream shortly after reticulocytes occur in increased numbers. Consequently, it seemed to us that perhaps the previously undetermined protoporphyrin, probably present in many of the highly parasitized blood samples, might account for the differences found in the ratios of the intracellular quinine concentration divided by the total pigment concentration.

From the results in Table VIII it is seen that, even in non-parasitized chicken blood, the I/E quinine ratio increases when the reticulocyte numbers also increase. Furthermore, in samples with high reticulocyte counts, there usually existed an increased

TABLE VIII

*Reticulocyte Counts, Free Protoporphyrin and Quinine Distribution*

Cells suspended in phosphate saline, pH 7.4; 15 minute incubation at 39°C.

In every case there were  $1.0 \pm 0.2 \times 10^9$  R.B.C./cc. of suspension.

Exp. no.	Type of infection	Reticulocyte count per cent of R. B. C.	P <sub>i</sub> Intracellular concentration of free protoporphyrin $M \times 10^{-10}$	H <sub>i</sub> Intracellular concentration of total pigment $M \times 10^{-4}$	Quinine distribution			$\frac{I}{P_i}$	$\frac{I}{H_i} \times 10^{-3}$
					I Intracellular concentration $M \times 10^{-10}$	E Extracellular concentration $M \times 10^{-10}$	$\frac{I}{E}$		
1	P (86)	3	6.02	10.6	7.82	2.60	30	13.0	7.36
2	A (29)	61	14.9	8.9	5.59	1.00	56	3.7	6.3
3	B (60)	6	2.09	13.4	7.15	2.42	30	34.2	5.4
4	B (51)	<1	1.06	14.2	5.03	2.33	22	47.5	3.5
5	A ( 0)	38	5.15	12.2	6.90	2.09	33	13.4	5.7
6	A (23)	72	8.24	9.8	5.95	1.50	40	7.2	6.1
7	A ( 0)	24	7.11	11.6	4.33	0.83	53	6.1	3.7
8	A (22)	44	6.44	11.3	5.58	1.41	40	8.7	5.0
9	A ( 0)	65	6.42	11.2	5.58	0.99	56	8.7	5.0
10	N	<1	2.02	15.3	5.02	3.27	15	24.8	3.3
11	N	<1	1.80	16.2	6.09	3.66	17	33.8	3.8
12	N	<1	1.47	15.0	5.88	3.59	16	40.0	3.9
13	N	<1	1.04	13.8	5.92	2.97	20	56.8	4.3
14	N	<1	0.89	13.5	5.72	3.37	17	64.3	4.2
15	N	<1	1.15	15.7	5.33	2.93	18	46.4	3.4
16	N	<1	1.55	15.4	4.96	3.66	14	32.0	3.2

protoporphyrin content, not, however, in direct proportion to the reticulocyte number. The results also indicate that, in every instance in which the normal I/E quinine ratio was exceeded, the protoporphyrin concentration was likewise found to be higher than normal. However, the increase in protoporphyrin content of these cells was not directly proportional to the increase in quinine.

To establish whether or not protoporphyrin actually is capable of altering the quinine distribution, protoporphyrin was added in varying amounts to our experimental system. The protoporphyrin used was prepared from hemin by the method

of Fischer and Putzer (17) and was purified as suggested by Grinstein and Watson (18).

The results of this experiment (Table IX) show that the addition of protoporphyrin had no effect on the quinine distribution. It may be noted that a considerable portion of the added protoporphyrin was no longer detectible as such and had probably been converted into a combined form.

TABLE IX

*Effect of Added Protoporphyrin on Quinine Distribution*

Normal blood cells suspended in phosphate-saline, pH 7.4; 15 minute incubation at 39°C.

In each case there were  $12.0 \times 10^9$  R.B.C. suspended in a final total volume of 12 cc.

The protoporphyrin was dissolved in phosphate-saline at pH 7.5.

Added protoporphyrin $\mu\text{g.} \times 10^{-2}$	Intracellular concentration of protoporphyrin $M \times 10^{-3}$	Extracellular concentration of protoporphyrin $M \times 10^{-3}$	Quinine distribution		
			I Intracellular concentration $M \times 10^{-3}$	E Extracellular concentration $M \times 10^{-3}$	$\frac{I}{E}$
0	1.39	0.017	5.19	3.96	13
10.7	2.06	0.149	5.61	3.89	14
21.4	2.83	0.307	5.79	4.02	14

## DISCUSSION

It is clear from the experiments of Oldham *et al.* (2), as well as from our own data, that the presence of the malaria parasite is not necessary for an increased intracellular concentration of quinine. However, this does not preclude the possibility that such an increased intracellular concentration of the drug is associated with the efficiency of its therapeutic action.

While it is evident that the quinine distribution can be modified by changes in pH of the suspending medium, and presumably of the intracellular contents, and is also affected by the concentrations of hemoglobin and the pigment resulting from its degradation (these being properties of the erythrocyte which are modified by the presence of the parasite), there is no direct relationship between any one of these factors and the intracellular concentration of quinine. Furthermore, it is not possible to state whether the unknown factor or factors responsible for the increased intracellular quinine concentration found in blood samples with high reticulocyte numbers are also involved in the case of parasitized cells.

## SUMMARY

1. In an experimental system consisting of normal and parasitized (*Plasmodium gallinaceum*) blood cells suspended in a phosphate-saline solution fortified with glucose, the  $\frac{\text{intracellular}}{\text{extracellular}}$  quinine ratio found with the parasitized samples usually exceeded that found with normal cells. This increased ratio was found not to be correlated with the parasitic type, the parasitic number or the parasitic surface area of the sample used. Nor was it related to the intracellular water content of the cells.

2. Changes in the composition of the suspending medium or the atmosphere also had no significant effect on the quinine distribution.

3. The pH of the medium apparently had the general effect of influencing the permeability of the cell membrane as, at the higher pH values, more quinine entered the cells. However, at the same pH the parasitized cells contained more quinine than did the normal cells.

4. There existed no direct relationship between any one of the blood pigments and the quinine distribution. However, both ferrihemic acid and, to a lesser extent, hemoglobin can alter the degree to which quinine enters the cell and, therefore, they very probably play an important role in the distribution of quinine between the cellular and extracellular components of our system.

5. The increased  $\frac{\text{intracellular}}{\text{extracellular}}$  quinine ratio is always found associated with a higher than normal reticulocyte count but is not directly proportional to this factor.

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# The Ultraviolet Absorption of the Plasma Proteins

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## INTRODUCTION

The first study of the ultraviolet absorption of proteins and amino acids apparently was made by Dhéré (1) in 1909. He showed that the selective absorption by proteins in the region of 2500–3000 Å was due to the aromatic amino acids phenylalanine, tyrosine and tryptophane. Since that time these observations have been verified by several investigators (2, 3, 4). It was later shown by Gróh and Hanák (5) and Holiday (6, 7) that the absorption of the proteins at 2800 Å could be accounted for by their tyrosine and tryptophane contents. This means that the molecular extinction coefficients of tyrosine and tryptophane are only slightly altered when their amino and carboxyl groups are tied into peptide linkages. Barkdoll and Ross (8) have recently shown that tyrosine, tyrosyltyrosine and tyrosyltyrosyltyrosine have approximately the same absorption per unit weight. Some believe that absorption of proteins for wave lengths below 2400 Å is due to the peptide linkage (9). However, this is probably not the case since biuret has only a very small absorption in that region.

Some workers hold that acid does not change the absorption of amino acids and proteins at 2800 Å (9). Strictly speaking, however, this is not true. As the pH of the solution is decreased, the maximum absorption shifts to shorter wave lengths.

Alkali brings about profound changes in the absorption of protein in the region 2400–3300 Å. The change may be explained partly by the ionization of the phenolic group of tyrosine (10, 11). Use has been made of the shift in tyrosine absorption in alkali to determine the concentration of both tryptophane and tyrosine in a number of proteins (12). This was possible because the absorption of tryptophane is only slightly changed by alkali. The absorption of the dissociated and undissociated forms of the phenolic group (of tyrosine) are independent of each other (3). Greenberg (9) has shown that, while the absorption of proteins is different in acids and in alkalis, heat denaturation does not appreciably alter the absorption. Enzymatic hydrolysis does not appreciably change the extinction coefficients of proteins at 2800 Å.

Recently plasma proteins have been separated on a large scale into five major fractions of known composition (13, 14). Each major fraction was divided into sub-fractions. Electrophoretic analyses were made on the preparation to determine the distribution of the various proteins.

In this paper a preliminary report of the ultraviolet absorption of some of these protein fractions is given.<sup>1</sup> Time did not permit a more detailed investigation, but the data obtained appeared to be of sufficient worth to merit publication.

### EXPERIMENTAL METHODS

All measurements were made with a Beckman quartz spectrophotometer with a hydrogen discharge tube and quartz cuvettes 1.00 cm. in thickness. Density readings were made every 25 Å except where maxima or minima occurred, in which case readings were made every 10 Å. The concentration of the solute was adjusted so that the densities of the solutions varied from 0.09 to 0.85.

Extinction coefficients were determined by the Beer-Lambert equation.

$$E = \frac{\log \frac{I_0}{I}}{cd}$$

where  $E$  is the extinction coefficient,  $\log I_0/I$  the density,  $c$  the concentration, and  $d$  the depth of the cell. When  $c$  is given in grams per 100 ml. and  $d$  equals 1 cm., the extinction coefficient is expressed as  $E_{1\%}^{1\text{cm.}}$ . When  $c$  is given in molarity, the molecular extinction coefficient is obtained.

*Absorption Spectra of the Plasma Protein Fractions.* With the exception of Fraction V, 25 mg. portions of the plasma protein fractions were dissolved in 0.01  $N$  HCl and 0.01  $N$  NaOH. Fraction V was treated in a similar manner, but 35 mg. were used. The final volumes were 50 ml.

The protein concentrations of the solutions were calculated from the nitrogen content by using the factor 6.25. Total nitrogen determinations were made by using the micro-Kjeldahl method described by Ma and Zuazaga (16). The final protein concentrations in mg.-% were Fraction I, 41.4; Fraction II-1, 45.4; Fraction II + III, 35.9; Fraction IV-1, 33.3; Fraction IV-3, 4, 38.8; and Fraction V, 64.6. The blanks were either 0.01  $N$  HCl or 0.01  $N$  NaOH solutions, depending upon whether the protein solutions were acid or alkaline.

*Absorption Spectra of the Aromatic Amino Acids.* In these experiments Eastman *l*-tryptophane, *dl*-phenylalanine, Hoffmann-La Roche *l*-dopa, and University of California *l*-tyrosine were used. All of the compounds were dried at 105°C. for 24 hours. All of the compounds were dissolved in 0.01  $N$  HCl. The concentrations of the final solutions in mg.-% were *l*-tryptophane, 2; *l*-tyrosine, 6; *l*-dopa, 4; *dl*-phenylalanine, 50. All of the blanks were 0.01  $N$  HCl solutions.

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<sup>1</sup> The plasma products employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

## DISCUSSION OF EXPERIMENTAL DATA

The ultraviolet absorption spectra of six plasma protein fractions were determined near pH 2 and 10. Below is a list of the compositions of the various fractions. The absorption curves for the fractions are given in Figs. 1, 2, 3, 4, 5 and 6.

Fraction I	60% fibrinogen
	19 $\beta$ -globulin
	12 $\gamma$ -globulin
	5 $\alpha$ -globulin
	5 albumin

Fraction II-1	98% $\gamma$ -globulin
	1 $\beta$ -globulin
	1 albumin

*plus* small amounts of cholesterol and phospholipids

Fraction II + III	38% $\beta$ -globulin
	37 $\gamma$ -globulin
	11 $\alpha$ -globulin
	10 fibrinogen
	4 albumin

*plus* 2/3 of the plasma cholesterol, carotinoids, phospholipids, Rh antibodies, isohemagglutinins and prothrombin

Fraction IV-1	39% albumin
	29 $\alpha_1$ -globulin
	22 $\alpha_2$ -globulin
	8 $\beta$ -globulin
	2 $\gamma$ -globulin

*plus* cholesterol and phospholipids, other lipids

Fraction IV-3, 4	39% $\beta_1$ -globulin
	2 $\beta_2$ -globulin
	16 $\alpha_1$ -globulin
	22 $\alpha_2$ -globulin
	20 albumin
	1 $\gamma$ -globulin

*plus* hypertensinogen and the thyrotropic hormones

Fraction V	99% albumin
	1 $\alpha$ -globulin

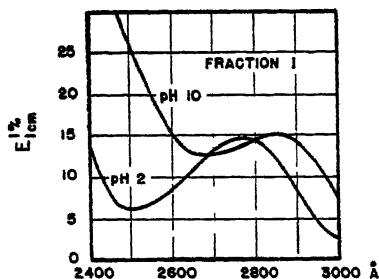


FIG 1

Absorption Spectra of Fraction I at  
pH 2 and pH 10

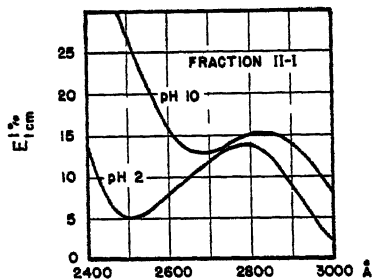


FIG 2

Absorption Spectra of Fraction II-I at  
pH 2 and pH 10

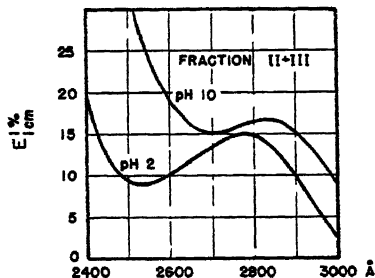


FIG 3

Absorption Spectra of Fraction II +  
III at pH 2 and pH 10

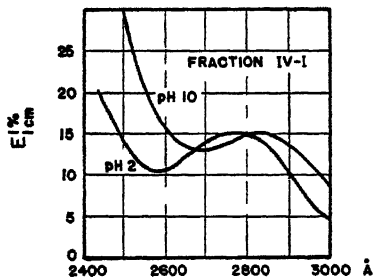


FIG 4

Absorption Spectra of Fraction IV-I at  
pH 2 and pH 10

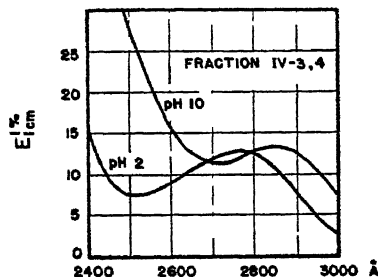


FIG 5

Absorption Spectra of Fraction IV-3, 4  
at pH 2 and pH 10

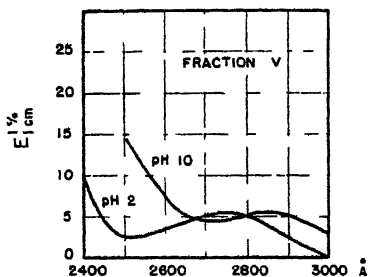


FIG 6

Absorption Spectra of Fraction V at  
pH 2 and pH 10

It will be noted that Fractions I, II + III, IV-1 and IV-3, 4 contain some albumin and  $\gamma$ -globulin. However, since the ultraviolet absorptions of albumin and  $\gamma$ -globulin were obtained from highly purified preparations, it should be possible to calculate the various absorptions of Fractions I, II + III, IV-1 and IV-3, 4 as if they were free from albumin and  $\gamma$ -globulin. In addition, it seemed possible to make a slight improvement in the absorption values for albumin by assuming that the 1% "impurities" of Fraction V was due to Fraction IV-1 (the fraction with the greatest amount of  $\alpha$ -globulin). The term "Recalculated Fraction" is given to a fraction when the albumin and  $\gamma$ -globulin have been removed by calculation. The compositions of the "Recalculated Fractions" are given below. Fig. 7 shows the absorption curves of albumin (Recalculated Fraction V),  $\gamma$ -globulin, and fibrinogen (Recalculated Fraction I). The absorption curves of the remaining "Recalculated Fractions" are given in Fig. 8.

Recalculated Fraction I	72% fibrinogen
	22 $\beta$ -globulin
	6 $\alpha$ -globulin
Fraction II-1	98% $\gamma$ -globulin
	1 albumin
	1 $\beta$ -globulin
Recalculated Fraction II + III	64% $\beta$ -globulin
	19 $\alpha$ -globulin
	17 fibrinogen
Recalculated Fraction IV-1	49% $\alpha_1$ -globulin
	37 $\alpha_2$ -globulin
	14 $\beta$ -globulin
Recalculated Fraction IV-3, 4	49% $\beta_1$ -globulin
	3 $\beta_2$ -globulin
	20 $\alpha_1$ -globulin
	28 $\alpha_2$ -globulin
Recalculated Fraction V	100% albumin

The calculations for the absorption of the "Recalculated Fractions" may not be valid because  $\alpha$ -,  $\beta$ - and  $\gamma$ -globulins and albumin are each made up of two or more proteins. This means that the various prep-

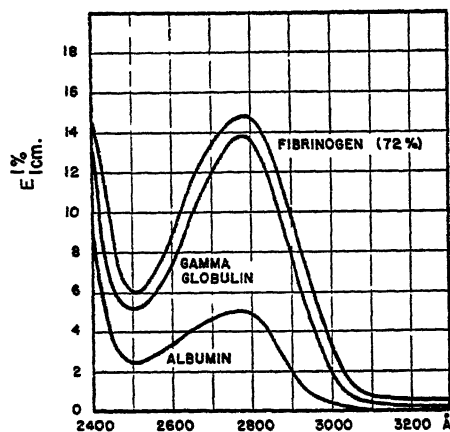


FIG. 7

Absorption Spectrum of  $\gamma$ -Globulin and Recalculated Absorption Spectrum Curves of Albumin and Fibrinogen at pH 2

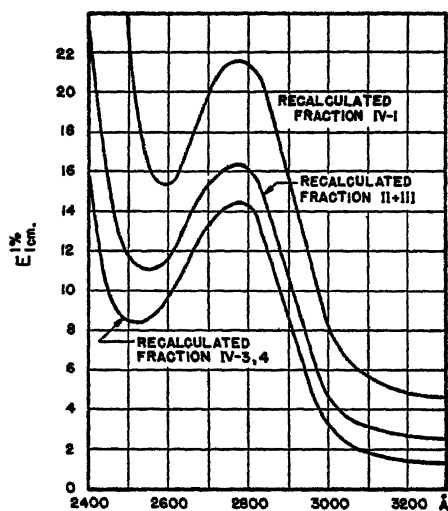


FIG. 8

Recalculated Absorption Spectrum Curves of Fraction II + III, IV-1, and IV-3, 4 at pH 2

arations probably contain different albumins and  $\gamma$ -globulins. The  $\gamma$ -globulin preparation used here is mainly pseudoglobulin, and there are indications that differences exist between the  $\gamma$ -euglobulin and pseudoglobulin (14). Therefore, it is possible that one cannot assume that all of the albumin in the various fractions has an absorption maximum of 5.11 and the  $\gamma$ -globulin a maximum of 13.9.

One should be careful in making any additional calculations to determine the absorptions of the  $\alpha$ - and  $\beta$ -globulins. However, it appears that the absorption of these two globulin groups is different. Fig. 8 shows the absorption curves for various mixtures of  $\alpha$ - and  $\beta$ -globulins. It must be noted that Fraction IV-3, 4 had a yellowish green color, and it was not possible to determine whether or not the coloring substance had an appreciable ultraviolet absorption.

*Theoretical Absorption Values.* If the ultraviolet absorption of proteins in the region 2400–3300 Å is due to the tyrosine and tryptophane in the protein molecule, it should be possible to calculate the absorption of a protein from values of the tyrosine and tryptophane content and the absorption of these amino acids. By using the data of Brand, Kassell and Saidel (15) on the amino acid compositions of some of these fractions, along with the absorption values of tyrosine and tryptophane shown in Fig. 9, the absorption maxima of some of the preparations in acid solution were calculated. The results are given in Table I.

It is obvious that the calculated and observed absorption values for albumin and  $\gamma$ -globulin are in close agreement. The calculated absorption value for fibrinogen was less than that observed, but part of this

TABLE I

Protein	Per cent Tyrosine	Per cent Tryptophane	$E_{1\text{ cm}}^{1\%}$ (calc) 2775 Å; pH 2	$E_{1\text{ cm}}^{1\%}$ (observed) 2775 Å; pH 2
Albumin	4.66	0.19	5.03	5.11
$\gamma$ -Globulin	6.75	2.86	13.30	13.88
Fibrinogen	5.75	3.29	13.78	14.85 (72% fibrinogen)
$\beta$ -Globulin concentrate*	5.60	2.06	10.12	

\*  $\beta$ -Globulin concentrate: 62%  $\beta$ -globulin  
11  $\alpha$ -globulin  
27  $\gamma$ -globulin



could be explained on the basis that the observed value was obtained from a not too pure fibrinogen preparation. The calculated absorption of a  $\beta$ -globulin concentrate indicates that  $\beta$ -globulin has the smallest absorption of the globulins.

Before it can be shown that tyrosine and tryptophane account for the absorption of proteins in the region 2400–3300 Å further investigations at a series of different pH values would have to be made.

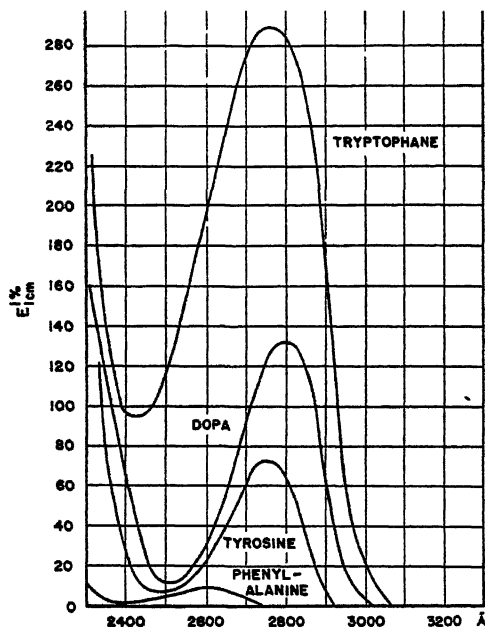


FIG. 9

Absorption Spectra of Tryptophane, Dopa, Tyrosine and Phenylalanine at pH 2

*Molecular Extinction Coefficients.* Until now the molecular extinction coefficients of proteins could not be calculated because the values of molecular weights and ultraviolet absorptions of proteins were not known with accuracy. However, by using the values given by Cohn *et al.* (14) on the molecular weights of the purified albumin and  $\gamma$ -globulin it is possible to calculate the molecular extinction coefficients in acid solution. The values obtained are given in the following table.

Molecular extinction coefficients 2775 Å; pH 2

Albumin	35,200
$\gamma$ -Globulin	216,000

## SUMMARY

The ultraviolet absorption curves of plasma protein fractions of known composition were obtained for acid and alkaline solutions. While the absorptions of the globulin fractions were somewhat similar, the albumin fraction was much lower.

Calculations were made to eliminate the albumin and  $\gamma$ -globulin absorption components in order to obtain curves for mixtures of fibrinogen and  $\alpha$ - and  $\beta$ -globulins.

The ultraviolet absorption spectra for tryptophane, dopa, tyrosine and phenylalanine were determined. Results were expressed as  $E_{1\text{cm}}^{1\%}$ .

The absorption maxima of albumin,  $\gamma$ -globulin, and fibrinogen were calculated from their tyrosine and tryptophane contents. These values are in good agreement with the observed maxima.

Molecular extinction coefficients for albumin and  $\gamma$ -globulin were calculated.

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# Note on the Use of Hydrogen Peroxide-Treated Peptone in Media for the Microbiological Determination of Amino Acids \*

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## INTRODUCTION

The preferential destruction or removal of certain amino acids from protein hydrolyzates or from partially hydrolyzed protein material such as peptone, offers the possibility of developing media for amino acid assays which are less expensive and also simpler to prepare than media which contain from 15 to 20 different pure amino acids. The medium used by Greene and Black (1) for the determination of tryptophane utilizes acid-hydrolyzed casein for this purpose, since hydrolysis with strong mineral acids results in the destruction of tryptophane.

The present communication reports the use of hydrogen peroxide-treated peptone in media for amino acid assays. Toennies and Callan (2) have studied the oxidation of methionine with hydrogen peroxide. Under the conditions given below, not only methionine, but also cystine, tryptophane and tyrosine are destroyed in peptone by treatment with hydrogen peroxide. Supplementation of the preparation with the four above mentioned amino acids results in a complete amino acid mixture for microbiological tests.

## EXPERIMENTAL

*Preparation of Hydrogen Peroxide-Treated Peptone.* Fifty g. of Bacto-Peptone in 500 ml. of 1 N HCl are treated with 0.05 mols of hydrogen peroxide (5.7 g. of 30%  $H_2O_2$ ) and allowed to stand overnight at room temperature. The solution is then

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heated in a steam sterilizer at atmospheric pressure for 30 minutes, cooled, neutralized with sodium hydroxide and steamed again, this time for one hour. The purpose of the second steaming is to decompose any hydrogen peroxide which is not used up by the oxidative reactions. The preparation is ready for use after diluting to a final volume of one liter.

In the experiments reported here, reagent grade of Superoxol which did not contain any preservative, was used. If the hydrogen peroxide without preservative has been stored for some time, it is advisable to standardize it before use. The treated peptone preparation may be kept in the ice box for several months if a little toluene is added.

*Composition of Assay Media.* The medium used for the tryptophane curve, with *Lactobacillus arabinosus* as the test organism, was prepared by replacing the amino acids in the medium of Kuiken *et al.* (3) with 50 mg. of hydrogen peroxide-treated peptone supplemented with 1 mg. each of methionine, cystine and tyrosine for each culture of 10 ml. volume.

The medium used for the tyrosine curve, using *Lactobacillus casei* as the test organism, was prepared by replacing the amino acids in the medium of McMahan and Snell (4) with 50 mg. of treated peptone as above. In this case the amino acid supplement consisted of 0.5 mg. of tryptophane and 1 mg. each of methionine and cystine for each culture of 10 ml. volume.

The medium used for the methionine curve, using *Leuconostoc mesenteroides*, was prepared by replacing the amino acid mixture in medium D of Dunn *et al.* (5) with 75 mg. of treated peptone per each culture of 10 ml. volume. The amino acid supplement was 0.5 mg. of tryptophane and 1 mg. each of cystine and tyrosine per tube.

Since the composition of the medium used for the curves using *Streptococcus faecalis* R has not been previously described, it is given in Table I. This organism is not as well adapted to growth in acid solution as are several of the other lactic acid bacteria which are used for amino acid assays, for example, *Lactobacillus arabinosus*. Sodium acetate is not the ideal buffer for this organism, because the range in which it acts as an efficient buffer is too far toward the acid side (ionization constant for acetic acid,  $1.75 \times 10^{-5}$ ). Sodium succinate acts as an efficient buffer in a range which is closer to neutrality (ionization constant for the second hydrogen ion,  $2.8 \times 10^{-6}$ ) and has been found to be a very satisfactory buffer for use with this organism. Five ml. of the basal medium given in Table I were used for each tube of final volume 10 ml.

*Procedures and Techniques.* The method of handling the organisms and of conducting the tests has been described in a previous publication (3).

## RESULTS AND DISCUSSION

The standard curves shown in Fig. 1, all of which were obtained with media in which hydrogen peroxide-treated peptone supplied most of the amino acid nitrogen, are typical of repeated tests. The slight dip in the methionine curve using *Leuconostoc mesenteroides* as the test organism always occurred in the methionine tests using this organism and this medium. A few attempts were made to obtain satisfactory cystine curves with media containing treated peptone. Because the data were

somewhat irregular and inconsistent, they are not included in this report.

The amount of hydrogen peroxide used in the preparation reported here is a little more than the minimum necessary to render methionine, tryptophane, tyrosine and cystine inactive in microbiological tests. This was done in order to allow for some variation in different batches of peptone. The exact amount of hydrogen peroxide required depends also on the acidity. Less  $H_2O_2$  is required in more acid solutions. A satisfactory peptone preparation for use in media for tryptophane

TABLE I

*Methionine Test Medium \* Used With Streptococcus faecalis R*

Glucose	20 g.	$p$ -Aminobenzoic acid	1 γ
$H_2O_2$ treated-peptone	5 g.	Xanthine	5 mg.
Succinic acid	10 g.	Folic acid (synthetic)	4 γ
Sodium acetate	3 g.	Pyridoxamine	0.2 mg.
Adenine sulfate	5 mg.	$K_2HPO_4$	0.5 g.
Guanine	5 mg.	$KH_2PO_4$	0.5 g.
Uracil	5 mg.	$MgSO_4 \cdot 7H_2O$	0.2 g.
Thiamine chloride	0.2 mg.	NaCl	0.01 g.
Pyridoxine	1 mg.	$MnSO_4 \cdot 4H_2O$	0.01 g.
Calcium pantothenate	0.2 mg.	$FeSO_4 \cdot 7H_2O$	0.01 g.
Biotin	1 γ	$l(-)$ -Cystine	0.1 g.
Riboflavin	0.5 mg.	$l(-)$ -Tyrosine	0.1 g.
Niacin	1 mg.	$l(-)$ -Tryptophane	0.05 g.

Neutralize with NaOH and dilute to a volume of 500 ml.

\* The tryptophane medium was prepared by omitting the tryptophane and adding 0.1 g. of *dl*-methionine.

determinations was obtained by the use of 0.4 *mM.*  $H_2O_2$ /g. of peptone in 0.5 *N* HCl solution. Under these conditions enough tyrosine is usually left in the peptone so that it is unnecessary to supplement the medium with this amino acid.

It has been the purpose of this communication merely to indicate the extent to which hydrogen peroxide-treated peptone may be used in media for amino acid assays. The results of analysis of proteins and foodstuffs will be given in a later paper.

The authors wish to express their appreciation to Lederle Laboratories, Inc., for the synthetic folic acid used in this investigation.

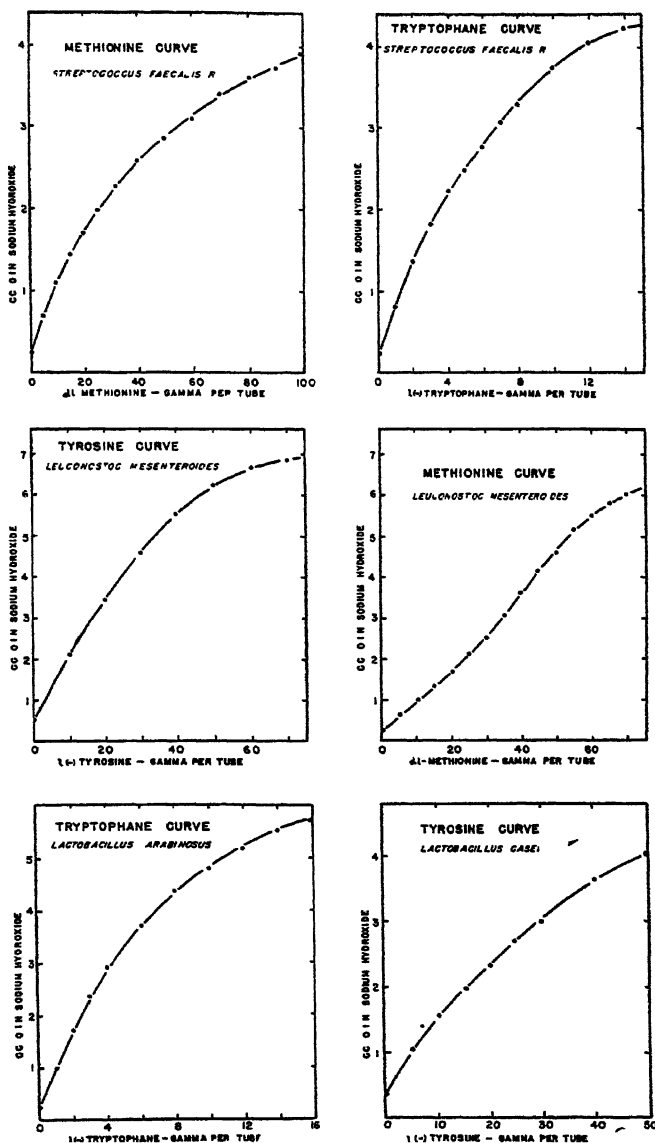


FIG. 1

Typical Standard Curves for Methionine, Tryptophane and Tyrosine, Using Several Microorganisms and Several Different Media Containing Hydrogen Peroxide-Treated Peptone as a Source of Amino Acids

Titration values are for 5 ml. aliquots from 10 ml. cultures. Incubation temperature, 35°C. Incubation time for *Leuconostoc mesenteroides*, 4 days; for other organisms, 3 days.

## SUMMARY

Conditions for the destruction of methionine, cystine, tryptophane and tyrosine in peptone by oxidation with hydrogen peroxide are given.

Smooth and regular standard curves for methionine, tryptophane and tyrosine were obtained with media in which most of the amino acid nitrogen was supplied by hydrogen peroxide-treated peptone.

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# Nutrition of Rainbow Trout; Studies with Purified Rations \*

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## INTRODUCTION

Studies on the nutrition of fish have been confined largely to the feeding of various natural materials. Although this work has resulted in the development of rations capable of producing as good, or better growth in the same species confined, as in the natural state, it has given little data on the exact requirements in terms of chemical compounds or even the major components of food, *i.e.*, fat, carbohydrate and protein. The studies reported here were planned to develop a ration of known composition so that the requirements in terms of chemical entities for growth and development of trout could be determined.

The earliest work with purified rations to assess the requirements of trout were the experiments performed by McCay (6). On these rations, fingerlings failed to grow even though various levels of protein, fat, carbohydrate and salts were tried, both in the presence and absence of the then known vitamin supplements. Further studies (16) established that fish receiving purified diets usually developed anemia before death. Hatchery practice has shown that trout could grow to maturity on a diet of fresh liver. McCay (7) postulated, therefore, the presence in liver of a substance (Factor H) distinct from the then known vitamins.

In a series of experiments carried out in 1940 (10) xanthopterin and a large number of liver fractions were found to be effective in curing the anemia. However, a year later difficulty was encountered in repeating these results (12) but in 1942 Cornell workers (13), under similar conditions, produced anemia which proved amenable to a combination of riboflavin, pyridoxine and pantothenic acid. They suggested that a combination of these three vitamins constitutes Factor "H." When attempts were made during the next year (15) to repeat this work, these vitamins failed to cause regeneration of the red blood cells.

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A serious complication in the use of synthetic diets in nutritional studies has been the method of feeding. If the ration is placed in the water it is impossible to accurately determine food consumption. Another complication is the leaching from the food of water-soluble substances. To overcome these difficulties, Field *et al.* (4) developed a method of hand-feeding gelatin capsules containing known quantities of ration.

### EXPERIMENTAL

All experiments were conducted in the biological laboratories of the James Nevni State Hatchery, Madison. Healthy yearling rainbow trout (*Salmo gairdnerii irideus*) were obtained directly from the stock raceways of the hatchery. The fish were selected to weigh 18–20 g. and divided into groups of 25. Each group was placed in a tank (13.5 × 1.5 × 1.5 feet) through which 60 gallons of fresh spring water flowed per hour. The average weights were obtained weekly using the method outlined by Field *et al.* (3).

In the first series seven groups were used. To all but one of these groups, dry diets were fed in capsules in an amount of 2% of their body weight per day. One group received fresh ground liver, dried at low temperatures. The other five groups were given synthetic diets based on a high sucrose ration consisting of: casein, 18; sucrose, 70; salts IV (9), 4; liver powder (1:20), 4; cod liver oil, 2; and corn oil, 2, supplemented with the following vitamins: thiamine, 0.3 mg.; riboflavin, 0.6 mg.; pantothenic acid, 1.5 mg.; nicotinic acid, 10.0 mg.; choline, 150.0 mg.; pyridoxine, 0.4 mg.; ascorbic acid, 50.0 mg.; biotin, 0.05 mg. and *p*-aminobenzoic acid, 0.05 mg. per 100 g. of ration. For a thiamine-free, ascorbic acid-free, liver-free and low protein ration this diet was modified by omitting or reducing respective factors.

The seventh, control group received an isocalorically equivalent amount (6%) of a diet consisting of fresh liver 47.5%, canned carp 47.5%, brewer's yeast 5%. This diet (Wisconsin Hatchery ration) has been shown to be more satisfactory than any combination of fresh food developed to date (8). This was fed by dropping into the tank.

The groups on the sucrose diets, but not those receiving the dried liver, regurgitated from 10–40% of their capsules after the first week, indicating a disturbance in the normal digestive processes. Over a ten week period, the control group which received the Wisconsin Hatchery ration gained an average of 3.3 g./week. The group receiving dried liver grew almost as well, averaging 3.1 g. gain/week. The group on the high sucrose diet grew poorly, averaging only 0.9 g./week. The deficient diets produced essentially the same growth as the high sucrose diet supplemented with all these factors, indicating that there was a limiting factor in the "complete" ration.

With these results in mind a new series was planned (Table I) in which two control groups, one receiving the standard hatchery diet, the other, dried liver plus 5% yeast was used. These were compared with three other diets prepared by adding increasing amounts of

TABLE I  
*Results of Feeding Trials with Different Levels of Carbohydrate*

Diet No.	Diet composition			Weekly gain (16 wks.)	Hemo- globin g./100 ml. blood	Liver wt. Body wt. × 100	Mortality No. of fish	Color of liver
	Dried liver	Yeast	Cerelose					
	Stock fresh meat			g				
				3.0	11.2	2.1	0	red
12	95	5	0	4.8	8.2	1.5	1	red
8	75	5	20	3.3	8.5	2.6	1	red
9	55	5	40	2.6	8.3	5.0	3	yellow
10	35	5	60	1.4	6.5	4.4	2	yellow

cerelose at the expense of the liver (diets 8, 9, 10). The fish receiving the dried liver-yeast diet gained an average of 4.8 g./week, as compared with 3.1 g. in the earlier series without the yeast. Substituting cerelose for the liver decreased growth but even at the 40% cerelose level the fish grew almost as well as those on the Wisconsin Hatchery ration. At 60% cerelose, growth was definitely poor. There was no significant mortality in any of these groups.

After the series summarized in Table I had been on experiment for 8 weeks, another series was designed to establish the optimum protein level using cerelose at 48%, yeast 5%, corn oil 2% and various levels of casein (Table II).

TABLE II  
*Effect of Different Levels of Protein*

Diet No.	Diet composition					Weekly gain (8 wks.)	Hemo- globin g./100 ml. blood	Liver wt. Body wt. × 100	Mortality No. of fish	Color of liver
	Dried liver	Yeast	Cere- lose	Casein	Corn oil					
						g.				
13	35	5	48	10	2	4.0	9.2	7.1	1	yellow
14	25	5	48	20	2	3.5	7.8	9.0	1	yellow
15	15	5	48	30	2	3.9	8.1	12.1	4	yellow
16	5	5	48	40	2	4.0	6.9	9.5	7	yellow

Growth on these four rations was very satisfactory but not as good as the 95% liver—5% yeast control group (diet no. 12) started earlier (Table II). On the 57th day it was noted that two fish receiving diet 16 (5% dried liver) had lost their equilibrium. Following feeding, their movements became even less coordinated, finally floating belly-up before dying the next day. An additional 5 fish died under similar circumstances in the course of the next 48 hours. On the 58th day, one fish from group 15, and during the succeeding days more fish from this group as well as those on diets 13 and 14, died. The stomachs of all these fish were distended and contained several undigested capsules often telescoped into each other. Autopsy also showed that they possessed extremely large, light-colored, lobulated livers. The livers accounted for approximately 10% of their body weight.

At least three fish from each of the groups listed in Table II, as well as those given in Table I, were bled for hemoglobin determination, then killed, and their livers removed and weighed. The hemoglobin values showed a slight decrease at the lower levels of liver and protein in the diet. The only fish with livers of color and size comparable with those receiving the Wisconsin Hatchery ration, taken here as a standard of normalcy, were those on diets 8 and 12.

The fish on diets 9 and 10 were continued on experiment with the same diet except that ground gelatin was added and food was fed by dropping into the tank. It required some time for the fish to adjust themselves to this new type of food. After a period of four weeks, they consumed it readily, although they never reached a daily consumption equal to 2% of their body weight. At the end of 12 months the liver to body weight ratio for three representative fish had been reduced from 5.0 to 2.6 for the group on 40% cerelese and from 4.3 to 1.7 on the 60% cerelese diet. It appears that the lessened food intake reduced the livers to normal size but they still retained, as would be expected, their lobulated shape. The hemoglobin values were within the normal range (2) although these fish had been given no fresh meat for a whole year.

Since it was possible that cerelese might be unique in its ability to cause abnormal livers, other carbohydrates were tested under similar conditions. Diet 15 of the previous series was used as a pattern. Starch, lactose, sucrose and dextrin and a carbohydrate mixture (equal amounts of starch, lactose and sucrose) was used at 28% with cerelese bringing the carbohydrate level up to 48% as shown in Table III.

TABLL III  
*Effect of Different Types of Carbohydrates*

Diet No.	Diet composition						Weekly gain (16 <sup>1</sup> wks.)	Hemo-globin g 100 ml blood	Liver wt Body wt × 100	Mor-tality No. of fish	Color of liver
	Dried liver	Yeast	Cere-lose	CHO	Casein	Corn oil					
	Stock standard			hatchery ration			g.				
15	15	5	48	0	30	2	1.2	13.8	1.8	0	red
21	15	5	20	28 <sup>1</sup>	30	2	1.3	10.8	2.0	1	yellow
22	15	5	20	28 <sup>1</sup>	30	2	1.5	12.0	1.7	2	yellow
23	15	5	20	28 <sup>1</sup>	30	2	1.7	8.6	1.6	2	yellow
24	15	5	20	28 <sup>1</sup>	30	2	1.2	10.8	2.5	7	yellow
26	15	5	20	28 <sup>1</sup>	30	2	1.4	10.3	2.0	2	yellow
	15	5	20	28 <sup>1</sup>	30	2	2.4	11.3	2.1	3	yellow

<sup>1</sup> Starch.

- Lactose

<sup>2</sup> Sucrose.<sup>1</sup> 9.3 g. each, starch, lactose and sucrose.<sup>3</sup> Dextrin

These fish grew rapidly at first but showed a plateau in growth rate about the sixth week and a few which died had three or four undigested capsules in their stomachs. The capsule size was reduced in all the groups in this series, and growth was then resumed but at a less rapid rate than in the previous period.

Groups on diets 21 and 23, starch and sucrose, even with reduced food intake, regurgitated their capsules. This is nearly always a sign of an undigested capsule in the stomach from a preceding feeding. When 0.3 mg.- $\%$  riboflavin, 0.4 mg.- $\%$  pyridoxine and 0.15% choline were added to these two rations regurgitation ceased, and growth approximated that of the other groups. Growth on all these rations, including the Wisconsin Hatchery ration, was much poorer than in the previous experiments. In order to get small fish at the time this experiment was started, it was necessary to use stunted yearlings which do not appear to grow so rapidly. All of the carbohydrate diets produced as good or better growth than the control diet. Indeed, the group on ration 26, dextrin, showed a rate of growth almost double that of the control. At 16 weeks representative fish were autopsied. In spite of the slight differences in growth all the fish on the high carbohydrate diets had livers which averaged 1.5-2.5% of the body weight, but were yellow in color and abnormal in shape. They appeared as if they had been enlarged and then regressed, possibly at the time the food intake was reduced. The addition of riboflavin, pyridoxine and choline appeared

to have aided digestion but not to have obviated liver damage. Hemoglobin values were within the normal range, although some groups were markedly below those of the fish receiving fresh meat.

Using stunted yearlings, which belonged to the same group described in Table III, another series (Table IV) was set up at the same time to

TABLE IV  
*Effect of Varying the Liver and Casein Content of the Ration*

Diet No.	Dried liver	Yeast	Cerelose	Casein	Corn oil	Weekly gain (16 wks.)	Hemoglobin g. 100 ml. blood	Liver wt. Body wt. $\times 100$	Mortality No. of fish	Color of liver
Stock	Wisconsin	Hatchery	diet			g.				
8	73	5	20	0	2	1.2 2.5	13.8 10.3	1.8 1.2	0 0	red red
17	0	5	20	73	0 <sup>1</sup>	2.7	7.5	2.5	13	yellow
18	5	5	20	68	2	1.9	8.6	1.2	0	red
19	15	5	20	58	2	1.9	7.3	1.3	2	red
20	25	5	20	48	2	2.1	10.9	1.2	0	red

<sup>1</sup> Contained 2% cod liver oil, 400 I.U. of vitamin D and 2000 units of vitamin A.

study the effect of dried liver. The control group received diet 8 modified to contain dried liver 73%, yeast 5% cerelose 20% and corn oil 2%. The carbohydrate was maintained at 20% and the liver replaced completely or in part by casein (diets 17-20). The group on diet 17 (no liver) grew as rapidly as those receiving liver for the first month but then they began to regurgitate their capsules. This group was divided into 2 parts and 12 fish were given supplements of riboflavin, pyridoxine and choline at the same levels as in the other series. This addition allowed the fish to digest their capsules and growth continued. The 13 fish left unsupplemented were all dead at the end of three weeks. The control group receiving 73% liver (diet 8) made the best growth, and the growth tended to be proportional to the amount of dried liver in the ration, except for the group on diet 17, where the addition of vitamins seemed to have replaced the liver as far as growth was concerned. Hemoglobin values were proportional to the amount of liver present, up to 15%. The vitamin supplementation seemed to be without effect on hemoglobin production. The livers were normal in color, shape and size in all of these groups receiving dried liver. Fish on diet 17, although supplemented with vitamins, had livers which were

yellow, slightly enlarged, lobulated with lumpy lighter yellow patches on the surface. This experiment was repeated with similar results. All the fish represented in Table IV received only 20% carbohydrate. In these the livers with the exception of those on diet 17 were essentially normal in size and appearance in contrast to the large number of fish on earlier experiments which had received levels of carbohydrate in excess of 20%.

### DISCUSSION

The failure of rainbow trout to grow on a casein-sucrose ration supplemented with mineral salts, vitamins and liver extract powder adequate for the rat or chick, confirms the early work of the Cornell group (6). In comparing the results recorded here it is well to bear in mind that we used yearlings, force-fed by capsule, whereas they employed fingerlings, self-fed.

The Wisconsin Hatchery diet, employed as a standard of reference here, produces as fast a growth rate as any hatchery ration reported (8). Liver dried at low temperatures, when fed in capsules, gave equally good growth responses. Addition of 5% yeast increases the growth slightly above that obtained with liver alone. When amounts of cerelese greater than 20% replaced the liver of this combination, the fish showed a reduced growth rate and abnormal livers. Growth could be maintained, even with 48% cerelese in the ration, if casein were present. Although the casein replaced an equal amount of liver, total protein in the diet was increased inasmuch as this liver preparation was only about 60% protein. This increase in protein is even greater if one uses a caloric basis of comparison, for the dried liver contained approximately 12% fat. At present, it would seem that it was the increased protein level rather than any specific factor in casein which maintained higher growth rates in diets 13-16 as compared with diet 9.

Sucrose, lactose, starch or a mixture of these three, appeared to produce essentially the same growth as does cerelese during the early growth with yearlings. Starch and sucrose rations required vitamin supplementation to carry them through the experimental period. With dextrin as the carbohydrate, growth was better with rainbow trout as it has been shown to be with other animals (5).

There is no clear-cut relationship between the level of dried liver and growth with diets of adequate protein content. Although it was unfortunate that only stunted yearlings were available for this experi-



ment, it will be seen from Table IV that the best growth of any of the casein-containing rations employed in that series was obtained on diet 17 which contained no liver. This evidence would seem to indicate that Factor H or other substances which may be present in liver are not essential for *growth* in yearling trout.

It must be recognized, however, especially in this species, that good growth is not the only criterion for adequate nutrition. Fish which made very satisfactory gains showed markedly abnormal livers and often an excessive and sudden mortality. It is evident from Tables I, II and III that fish receiving more than 20% carbohydrate showed liver pathology and increased mortality regardless of their rate of growth. Indeed, with faster growing fish they seemed to have more severely enlarged livers as can be noted from Table II. Embury's analysis of stomachs of wild brook trout show that their natural diet probably contains about 20% carbohydrate (1). Liver pathology is evident whenever a high level of carbohydrate is used. With these high levels, the type of carbohydrate and amount of protein has no beneficial or detrimental effect. Furthermore, the level of liver is without effect as long as the carbohydrate level is about 20%. On low protein, better than 50% dried liver is necessary to prevent this pathology (Table I). When the carbohydrate is low and the protein is more nearly adequate, as in the series presented in Table IV, an amount of dried liver as small as 5% appears to prevent both mortality and liver damage. Carefully dried liver seems to contain a factor (possibly Factor H), or factors, necessary for yearling rainbow trout fed a purified casein-carbohydrate-yeast ration. It is apparent that this factor is not riboflavin, pyridoxine or choline as these were fed in combination, at the level found adequate for the chick to one-half the group on diet 17 (adequate protein—no liver), without remedying the liver pathology. The fish which received these vitamins lived through the experimental period whereas those which received none, all died. Part, but only part, of the beneficial effect of dried liver would be in its content of these vitamins.

In contrast to McCay's results (15) we have not noted any pronounced anemias. Perhaps this may be due to our use of larger and more mature fish. Hemoglobin values have been independent of growth or liver pathology except possibly in series presented in Table IV where, there may be some positive correlation between hemoglobin values and the percentage of dried liver fed.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the generous supply of crystalline B vitamins and to Wilson Laboratories, Inc., Chicago, Illinois, for liver preparation.

### SUMMARY

1. Yearling rainbow trout fed a ration composed of cerelose 48%, casein 40%, fat 2%, supplemented with 5% each of dried liver and brewers' yeast by capsule, were able to maintain a hemoglobin level and growth rate equal to that produced by feeding 100% dried liver or a standard hatchery meat ration.

2. Such fish develop greatly enlarged yellow, lobulated livers and die suddenly after 8 weeks on experiment.

3. To prevent liver damage it was necessary to cut the carbohydrate level to 20% and maintain the level of liver at 5%.

4. Part, but not all, of the effect of liver can be replaced by a combination of riboflavin, pyridoxine and choline.

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## Enzymatic Oxidation of Glutathione II. Studies on the Addition of Several Cofactors \*

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### INTRODUCTION

In a previous publication (1), we reported that reduced glutathione is rapidly oxidized by a cytochrome c-linked enzymatic system found in cell-free preparations of mouse kidney homogenates. In order to determine whether the system was complete, as previously described, a study was made on the effect of the addition of several co-factors, such as coenzyme I, nicotinamide, ascorbic acid and adenosine triphosphate (ATP).

Little work has been done relating glutathione and coenzyme I in animal tissues. Bukin (2) has observed a non-enzymatic reaction in which oxidized glutathione is reduced by dihydrocozymase.

A relationship not as yet completely understood seems to exist between glutathione and ascorbic acid. Hopkins and Morgan (3) observed that, in the presence of crude ascorbic acid oxidase, any glutathione present was completely oxidized before the ascorbic acid itself was oxidized. In addition, reduced glutathione could reduce oxidized ascorbic acid very rapidly if the enzyme was present. Crook (4) has separated the enzyme, catalyzing the reduction of dehydroascorbic acid by glutathione, from its associated ascorbic acid oxidase.

Bukin (2) related ascorbic acid, glutathione and dihydrocozymase in plant systems and concluded that one of the main functions of ascorbic acid in plant tissues depends on its participation in the respiration process. However, he indicated that this is not the case in animal cells. Stotz *et al.* (5) observed that reduced glutathione disappeared from liver *brei* at essentially the same rate whether or not ascorbic acid was added. On the basis of this and other observations, they concluded that ascorbic acid was not an essential intermediate in the reaction of glutathione with oxygen, either through the indophenol oxidase-cytochrome system or in the presence of copper.

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In this study, data are presented which indicate that coenzyme I and ascorbic acid are involved in the enzymatic oxidation of reduced glutathione and the effects of these and other cofactors are discussed.

## METHODS

White mice of an inbred Swiss strain were used and, after weaning, were maintained on stock ration<sup>1</sup> and water *ad libitum* plus occasional greens. A tissue homogenate of mouse kidney was prepared by the technique of Potter and Elvehjem (6) modified as previously described (7, 8). From this homogenate a cell-free preparation (1) was obtained which was used immediately in order to minimize inactivation of the enzyme occurring in very dilute solutions of protein (9).

A conventional Warburg constant volume respirometer at 37.0°C. was used in all experimental and analytical work and the pH was determined with a Beckman pH meter (glass electrode). The reaction was buffered in all cases with 0.4 ml. of 0.25 *M* sodium potassium phosphate buffer (pH 7.6)<sup>2</sup> and, after adding the other components, redistilled water was added to make 3.0 ml. The gas phase was air, and 0.2 ml. of 10% KOH and a small strip of filter paper were placed in the center well to absorb CO<sub>2</sub>. The pH of the final reaction mixture was 7.6 as determined electrometrically. The glutathione solution was always placed in a side arm and added to the mixture of the other components after equilibration of the flask contents had taken place.

Commercially prepared compounds were used without further purification as follows: ascorbic acid, Merck; U.S.P. nicotinamide, Merck; commercially isolated coenzyme I (86% pure), Merck;<sup>3</sup> and pure, crystalline, reduced glutathione, Eastman Kodak and B. L. Lemke. Adenosine triphosphate was isolated in these laboratories<sup>4</sup> from dog muscle by a procedure essentially as described by Le Page (10) and a semipurified fraction (86% purity) was used in these experiments. Cytochrome c was prepared in these laboratories from beef heart by the method of Keilin and Hartree (11) and had been dialyzed against glass-redistilled water. All solutions were prepared in glass-redistilled water and neutralized, if necessary, to pH 7.6 with dilute sodium hydroxide. Glutathione and ascorbic acid solutions were neutralized just before addition to the side arm of the reaction vessel.

## EXPERIMENTAL

In addition to the rapid enzymatic oxidation of glutathione occurring when cytochrome c was added, it was previously observed (1) that

<sup>1</sup> B-B Laboratory Rabbit Diet, Maritime Milling Company, Inc., Buffalo, New York.

<sup>2</sup> This buffer was prepared by mixing 0.25 *M* disodium phosphate (six volumes) and 0.25 *M* monopotassium phosphate (one volume) until a pH of 7.6 was obtained when diluted to 0.033 *M*.

<sup>3</sup> We are indebted to Merck and Company, Inc., Rahway, New Jersey, for a generous gift of cozymase.

<sup>4</sup> Appreciation is expressed to F. J. Pilgrim who assisted in the isolation of ATP.

reduced glutathione was slowly oxidized by tissue preparations in the absence of cytochrome c. The effect of the addition of several cofactors was determined experimentally both in the presence and in the absence of cytochrome c and also on the non-enzymatic autoxidation of reduced glutathione.

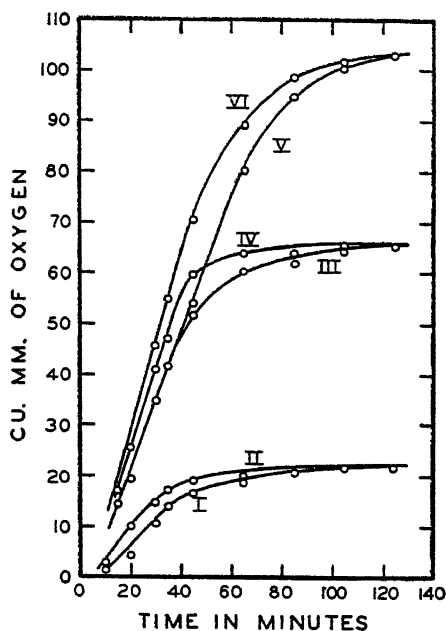


FIG. 1

Effect of Coenzyme I, Nicotinamide and Cytochrome c on the Oxidation of Glutathione by Tissue Homogenates

- Curve I, buffer, cytochrome c, tissue homogenate representing 20 mg. wet weight of mouse kidney (dry weight of about 4 mg.), and 1 mg. of reduced glutathione;  
 Curve II, same as Curve I with addition of coenzyme I and nicotinamide;  
 Curve III, same as Curve I, except 3 mg. of glutathione;  
 Curve IV, same as Curve III with addition of coenzyme I and nicotinamide;  
 Curve V, same as Curve I, except 5 mg. of glutathione;  
 Curve VI, same as Curve V with addition of coenzyme I and nicotinamide.

Other final concentrations were buffer, 0.033 *M* potassium phosphate (pH 7.6); cytochrome c,  $4.7 \times 10^{-6}$  *M*; coenzyme I, 1 mg./flask; and nicotinamide,  $10^{-3}$  *M*. Redistilled water was added to make a total volume of 3.0 ml. The tissue homogenate was added, the flasks equilibrated and the glutathione solution was added at zero time from the side arm. All curves are corrected for the endogenous respiration occurring under the same conditions but in the absence of glutathione.

It became apparent early in our work that there was some inter-relationship between coenzyme I and glutathione oxidation. Hence, studies were made on the effect of addition of coenzyme I and nicotinamide to the reaction mixture containing cytochrome *c* and tissue homogenate. On the addition of reduced glutathione at three different concentrations, a small but definite increase in the rate of oxidation was obtained in each case (Fig. 1) when coenzyme I and nicotinamide were present. However, the values for the maximum oxygen uptake were the same, irrespective of further addition of coenzyme I and nicotinamide.

The results of the addition of cell-free tissue preparation to reduced glutathione in the presence of coenzyme I but in the absence of cytochrome *c* are summarized in Fig. 2. Furthermore, the additional effects which could be attributed to nicotinamide and ATP were investigated. The addition of coenzyme I at a level of 1 mg. usually caused a large increase in the rate of oxidation over the rate when no coenzyme I was added (Curve III, Fig. 2). In a few cases in which the addition of coenzyme I did not cause a marked increase in the oxidation rate, an increased rate of oxidation was obtained when both coenzyme I and nicotinamide were present. However, when an initial increase was obtained on the addition of coenzyme I alone, only a slight increase was observed on the further addition of nicotinamide (Curve IV, Fig. 2). These results can be explained by the variation in ability to destroy coenzyme I between different animals and the sparing action of nicotinamide on the inactivation of coenzyme I by the nucleotidases present. It will be noted that, in addition to the increase in the rate of oxidation observed when nicotinamide and coenzyme I are added, there is also an observable decrease in the induction period. In order to establish whether nicotinamide was exerting its effect in addition to or in conjunction with coenzyme I, it was added alone to an enzyme-catalyzed oxidation of glutathione. No increase was observed (Curve V, Fig. 2), and the same result was obtained when ATP was tried in a similar manner (Curve VI, Fig. 2). In no case did the rate of oxidation achieved by adding coenzyme I approach that which could be obtained on the addition of cytochrome *c*.

The addition of coenzyme I to a reaction mixture which contains tissue preparation, glutathione, and cytochrome *c* does not result in as striking a change as when cytochrome *c* is not present. The presence of coenzyme I and nicotinamide does not increase the rate of gluta-

thione oxidation but does significantly shorten the induction period (Curves VIII and IX, Fig. 2). As previously observed in the absence of cytochrome *c*, in some cases the addition of nicotinamide showed no effect over the addition of coenzyme I alone, whereas in some cases its presence was necessary before the effects attributed to the presence of

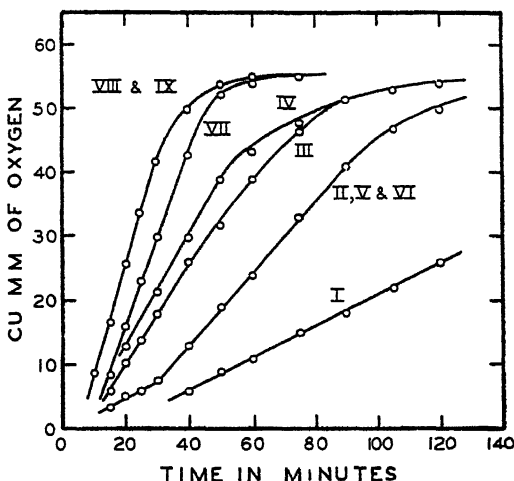


Fig. 2

Effect of Coenzyme I and Other Cofactors on the Oxidation of Glutathione by Cell-Free Tissue Preparations

- Curve I, buffer only or buffer and cytochrome *c*;  
 Curve II, buffer and cell-free tissue preparation representing 2.0 mg. wet weight of mouse kidney (dry weight of about 0.4 mg.);  
 Curve III, same as Curve II with addition of coenzyme I;  
 Curve IV, same as Curve II with addition of coenzyme I and nicotinamide;  
 Curve V, same as Curve II with addition of nicotinamide (identical with Curve II).  
 Curve VI, same as Curve II with addition of ATP (identical with Curve II);  
 Curve VII, same as Curve II with addition of cytochrome *c*;  
 Curve VIII, same as Curve VII with addition of coenzyme I;  
 Curve IX, same as Curve VII with addition of coenzyme I, nicotinamide and ATP (identical with Curve VIII).

Reduced glutathione was present in all cases at a level of 3 mg./flask. Other final concentrations were buffer, 0.033 *M* sodium potassium phosphate (pH 7.6); cytochrome *c*,  $6.7 \times 10^{-6}$  *M*; coenzyme I, 1 mg./flask; nicotinamide,  $10^{-3}$  *M*; and ATP,  $1.33 \times 10^{-3}$  *M*. Redistilled water was added to make a total volume of 3.0 ml. The tissue preparation was added, the flasks equilibrated and the glutathione solution was added at zero time from the side arm.



coenzyme I were apparent. As when observed in the absence of cytochrome c, the addition of ATP was without effect either in the presence or absence of coenzyme I and nicotinamide.

The effects of the addition of several cofactors on the non-enzymatic reactions involved in the oxidation of reduced glutathione were either no change or a very slight increase in oxidation rate when these substances were added either singly or together (Table I). In general,

TABLE I  
*Effect of Coenzyme I on the Non-Enzymatic Oxidation of Glutathione*

Exp.	Contents of Flask	Rate of O <sub>2</sub> Uptake (cu mm hr)
1	GSH with or without cyto. c	16
2	GSH, Co. I (1 mg.)	17, 25
3	GSH, Co. I (1 mg.), NA, cyto. c	20, 23, 25
4	GSH, Co. I (1 mg.), NA, cyto. c, ATP	24
5	GSH, Co. I (0.2 mg.)	17
6	GSH, Co. I (0.6 mg.)	17
7	GSH, Co. I (2 mg.)	16

Reduced glutathione (GSH) at a level of 3 mg. per flask and buffer, 0.033 *M* sodium potassium phosphate (pH 7.6), were present in all experiments. Other final concentrations were coenzyme I (Co. I), level indicated/flask; cytochrome c (cyto. c),  $6.7 \times 10^{-6}$  *M*; nicotinamide (NA),  $10^{-2}$  *M*; and adenosine triphosphate (ATP),  $1.33 \times 10^{-3}$  *M*. Tissue preparation was not present. Redistilled water was added to make a total volume of 3.0 ml., the flasks were equilibrated, and glutathione solution was added at zero time from the side arm.

In most cases, each value for the rate of O<sub>2</sub> uptake represents an average of several experiments.

the rate of non-enzymatic oxidation of glutathione is considered to be unaltered by the addition of cytochrome c, coenzyme I, nicotinamide or ATP.

It is known that ascorbic acid can be used as a substrate in the determination of cytochrome oxidase (12) and that it is non-enzymatically oxidized in the presence of heavy metals such as copper (13). In these experiments the rate of oxidation of ascorbic acid in the absence of glutathione was determined in order to ascertain whether or not this oxidation rate was sufficiently large to account for the differences observed when glutathione was present. On the addition of a cell-free preparation of mouse kidney, ascorbic acid is oxidized

but at a very low rate as indicated in Table II. When cytochrome c is added, but in the absence of tissue preparation, the oxidation of ascorbic acid proceeds more rapidly, but still at a very slow rate. When both cytochrome c and tissue preparation are added, ascorbic acid is oxidized at a rate which is approximately the summation of the rates observed when these two substances are added separately.

TABLE II  
*Effect of Ascorbic Acid on Glutathione Oxidation*

Exp.	Contents of Flask	Rate of O <sub>2</sub> Uptake (cu. mm/hr.)	QO <sub>2</sub>
1	AA, CFP	5	13
2	AA, cyto. c	10	—
3	AA, cyto. c, CFP	18	45
4	GSH, CFP	35	87
5	GSH, cyto. c	15	—
6	GSH, cyto. c, CFP	90	225
7	GSH, AA	26	—
8	GSH, AA, cyto. c	24	—
9	GSH, AA, CFP	60	150
10	GSH, AA, cyto. c, CFP	120	300
11	AA (low level), GSH, cyto. c, CFP	123	307
12	AA (high level), GSH, cyto. c, CFP	126	315

Buffer, 0.033 *M* sodium potassium phosphate (pH 7.6), was present in all experiments. Other final concentrations were reduced glutathione (GSH), 3 mg./flask; ascorbic acid (AA),  $10^{-3}$  *M*; low level of ascorbic acid (Exp. 11),  $1.7 \times 10^{-5}$  *M*; high level of ascorbic acid (Exp. 12),  $3.3 \times 10^{-3}$  *M*; cytochrome c (cyto. c),  $6.7 \times 10^{-5}$  *M*; and cell-free tissue preparation (CFP) representing 2.0 mg. wet weight of mouse kidney (dry weight of about 0.4 mg.). Redistilled water was added to make a total volume of 3.0 ml. The tissue preparation was added (where designated), the flasks equilibrated and the ascorbic acid and/or glutathione solutions were added at zero time from separate side arms.

In most cases, each value for the rate of O<sub>2</sub> uptake represents an average of several experiments.

When ascorbic acid and reduced glutathione are present together in a reaction vessel in the absence of cytochrome c or tissue preparation, the oxidation rate which results can be accounted for by a summation of the oxidation rates observed when each of these components is present separately (Table II). From these data, the conclusion can be drawn that ascorbic acid does not exert a non-enzymatic catalytic action on the rate of oxidation of reduced glutathione.

In the absence of cytochrome *c*, an enzymatic oxidation of both reduced glutathione and ascorbic acid takes place, but the latter at a much slower rate. When ascorbic acid and glutathione are both present under the same conditions as before, an oxidation occurs in which the rate exceeds by 50% the summation of the oxidation rates of ascorbic acid and glutathione when present separately under the same conditions. These data, summarized in Table II, indicate that ascorbic acid and glutathione interact to promote a more rapid oxygen uptake when tissue preparation is present.

The addition of ascorbic acid to a reaction mixture containing glutathione, cytochrome *c* and tissue preparation results in an oxidation rate which exceeds that of the summation of each of the oxidation rates of the two hydrogen donors considered separately (Table II). However, it will be noted that this relationship could very easily be overlooked if the amount of ascorbic acid added were too high. Apparently a very minute amount of ascorbic acid ( $1.7 \times 10^{-5}$  *M*) will cause a marked increase in the oxidation rate of reduced glutathione and increasing this amount by 200 times does not result in any further increase. A consideration of ten different experiments with amounts of added ascorbic acid differing by 200-fold results in an average deviation of approximately  $\pm 1\%$ , which is well within the experimental error of the manometric method.

The effect of variation in concentration of cell-free tissue preparation was investigated over a 40-fold range and the results are recorded in Table III. It is evident that both the rate and the total oxygen uptake increase as more tissue is added. The proportional increase does not seem to be a direct function of the amount of tissue added, particularly when the rate of the reaction is considered. Calculations of the  $Q_{O_2}$ , after correction has been made for the oxidation in the absence of tissue, show a progressive decrease from 205 to 8 as increasing amounts of tissue are added. On the other hand, by applying a correction obtained in the same concentration of substrates in the absence of tissue preparation, it will be observed that the total oxygen uptake is now a function of the amount of tissue added and lies in the neighborhood of 10 cu. mm./mg. This oxygen uptake is considerably larger than that previously obtained in the absence of ascorbic acid (1) and, therefore, it would seem that this compound perhaps facilitates in some manner oxidation of either protein sulfhydryl groups or substrates already present in the tissue preparation.

TABLE III

*Effect of Tissue Level on Glutathione Oxidation in the Presence of Ascorbic Acid*

Exp.	Tissue added (dry wt. in mg.)	Rate of O <sub>2</sub> Uptake (cu. mm./hr.)	Total O <sub>2</sub> Uptake (cu. mm.)	Total O <sub>2</sub> Uptake (cu. mm./mg.)
1	0	23	93	—
2	0.2	100	—	—
3	0.4	116	98	12
4	1.0	136	104	11
5	1.6	144	—	—
6	2.0	148	118	12.5
7	4.0	148	134	10.2
8	7.6	156	148	7.3

Each reaction flask contained buffer, 0.033 *M* sodium potassium phosphate (pH 7.6); cytochrome *c*,  $6.7 \times 10^{-6}$  *M*; ascorbic acid,  $10^{-3}$  *M*; glutathione, 3 mg.; and cell-free preparation of mouse kidney (20% dry weight) at the level indicated. Redistilled water was added to make a total of 3.0 ml. After addition of the cell-free preparation, the flasks were equilibrated and the ascorbic acid and glutathione added simultaneously at zero time from separate side arms.

The O<sub>2</sub> uptake was recorded over a total of 7 hours. The values in the last column were obtained by subtracting the total O<sub>2</sub> uptake obtained when no tissue preparation was added and then dividing by the number of mg. of tissue present.

## DISCUSSION

It is evident from the data relating coenzyme I and reduced glutathione, that the non-enzymatic oxidation is not stimulated by the addition of coenzyme I. When tissue preparations are added in addition to coenzyme I, the oxidation of glutathione occurs at a more rapid rate than in the absence of the cofactor, indicating that, in the absence of cytochrome *c*, coenzyme I is apparently a limiting factor in the oxidation of glutathione by tissue preparations. It can be concluded, therefore, that the oxidation of glutathione in the absence of cytochrome *c* probably involves a coenzyme I-linked enzyme system. When cytochrome *c* is present the effects attributed to coenzyme I are not as marked as before; the induction period is decreased but the rate remains the same. These results would suggest that either the cytochrome *c*-linked system does not need coenzyme I or that a sufficient quantity is already present. In the presence of cytochrome *c* the effects observed on the addition of coenzyme I are probably only of secondary nature.

The experiments involving the addition of ATP were performed in order to ascertain whether the oxidation of glutathione involved a phosphorylating mechanism which could be rendered more active on the addition of a donor of energy-rich phosphate bonds (14). No increases in oxidation are observed on the addition of ATP, either in the presence or absence of cytochrome c, showing that under these conditions there are no indications that the mechanism for glutathione oxidation involves phosphorylation in an intermediate step.

Ascorbic acid is shown to stimulate the rate of oxidation of reduced glutathione by cell-free preparations both in the presence and in the absence of cytochrome c. This is a catalytic effect and, after a small quantity of ascorbic acid (final concentration of  $1.7 \times 10^{-5} M$  or less) is added, no increase in rate is noted on further addition of ascorbic acid. These observations extend to animal tissues the conclusions of former investigators relating the oxidation of ascorbic acid and glutathione and a possible explanation of these effects is that ascorbic acid is functioning as a hydrogen carrier in glutathione oxidation. Furthermore, the presence of ascorbic acid results in an increase in the total oxygen uptake per mg. dry weight of tissue preparation, indicating that it might in some manner facilitate the oxidation of fixed sulfhydryls in tissue proteins.

### SUMMARY

1. Coenzyme I is shown to increase the rate of enzymatic oxidation of reduced glutathione in the absence of cytochrome c and to decrease the induction period either in the presence or absence of cytochrome c.

2. The addition of adenosine triphosphate, either in the presence or absence of cytochrome c, has no effect on the enzymatic oxidation of reduced glutathione.

3. Ascorbic acid catalytically stimulates the oxidation of reduced glutathione by cell-free tissue preparations both in the presence and absence of cytochrome c.

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# Glutamic Acid Decarboxylase of Higher Plants.

## I. Distribution; Preparation of Clear Solutions; Nature of Prosthetic Group

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### INTRODUCTION

Okunuki (1) discovered in a variety of higher plants an enzyme which specifically decarboxylated glutamic acid. He described glutamic acid decarboxylase as bound to the cellular structures from which it could not be brought into solution by treatment with glycerol (60%), phosphate buffers ( $m/10$  to  $m/40$ , pH 6.0 and 8.0) and NaCl solution. The activity of Okunuki's enzyme suspensions was not influenced by the presence or absence of oxygen or by treatment with carbon monoxide. Cyanide, however, inhibited 89% at a concentration of  $10^{-4}$  *M* and 100% at  $10^{-3}$  *M* and pH 6.0. Optimal activity of the enzyme was stated to occur near pH 6.0.

Okunuki's richest enzyme source was pollen of *Lilium auratum*. Cabbage contained about 1/2 as much enzyme, spinach 1/5 and carrots between 1/7 and 1/8, as judged from the amounts of CO<sub>2</sub> liberated in 30 min. by the action of 100 mg. of dry powder prepared from the sources mentioned.

In the experiments to be reported here (2) a simple method is described for the preparation of clear solutions of glutamic acid decarboxylase from carrots. A number of properties of such solutions were studied and evidence was obtained which suggested that the enzyme is a pyridoxal-phosphate-protein complex.

### EXPERIMENTAL

#### 1. Measurement of Enzymatic Activity

The decarboxylation of glutamic acid was followed manometrically with Warburg manometers at 37°C. The main vessel contained the enzyme suspension or solution



to be tested, buffered to pH 5.70–5.90 (glass electrode) with *m*/15 phosphate buffer. After temperature equilibrium had been reached, 0.5 ml. (34 micromol) of a neutral 1.000% solution of *l*(+)-glutamic acid was added from the first side vessel. (A solution of 1000 mg. glutamic acid (6.8 millimol) plus 6.2 ml. 1.095 *N* NaOH (6.8 millimol) filled with water up to 100 ml. had a pH of 7.04).

The volume of the enzyme-substrate mixture was usually 4.5 ml., so that the substrate concentration was 7.55 millimol/l. at the start of the reaction. Readings were taken at intervals, usually every ten minutes, and the reaction was usually terminated by the addition of 0.5 ml. 1.2 *N* H<sub>2</sub>SO<sub>4</sub> from the second side vessel. The individual readings were then corrected for "bound" CO<sub>2</sub>, liberated after the addition of acid. A blank was set up with each run containing water instead of glutamic acid solution and a correction for small amounts of CO<sub>2</sub> formed in these controls and contained in the buffers was made.

All experiments were carried out in nitrogen atmosphere. The main reason for this is the elimination of oxygen uptake, which, in air, amounted to about 5–15% of the CO<sub>2</sub> produced and can add confusing side reactions to the decarboxylation to be studied. The CO<sub>2</sub> formation in air in our experiments with carrots was 90–95% of that observed in nitrogen, whereas Okunuki (1) found 98% in an experiment with pollen of *Lilium auratum*.

## 2. Distribution of Glutamic Acid Decarboxylase in Various Plants

Suspensions of a variety of plants were prepared by mixing from 4–20 g. of plant material for 2–3 min. in a Waring Blendor with 100 ml. ice cold *m*/15 phosphate buffer pH 5.75. The tests for enzymatic activity were carried out with 4 ml. of each suspension. The results obtained with 34 plants are listed in Table I. Activities are recorded there as  $Q_{CO_2}^{N_2}$  values, obtained by multiplying mm.<sup>3</sup> CO<sub>2</sub> formed in 10 min. by 6 and dividing by the mg. of tissue used in each assay. Such *Q* values do not represent initial velocities and are to be considered as minimal values, as some inactivation of the enzyme has already taken place during the first 10 min. As the process of inactivation progresses rapidly, the actual amount of CO<sub>2</sub> liberated/mg. tissue/hr. is again smaller than would be expected from the *Q* values. Whereas *Q*<sub>CO<sub>2</sub></sub> (10 min.) is, therefore, a somewhat artificial concept—the multiplication with 6 is carried out to conform with the customary definition of *Q* values—it is, nevertheless, convenient for comparisons, particularly as values calculated in a similar way have been used by Blaschko (3) in his review dealing with amino acid decarboxylases of mammalian tissue.

Examination of Table I reveals that carrots are a fair source for glutamic acid decarboxylase. The discrepancy with the results obtained by Okunuki may be due to the fact that he washed his suspensions, removed as much fluid as possible by strong pressing and assayed dry powders prepared from the residual tissue. In

Okunuki's report, the amounts of  $\text{CO}_2$  formed by 100 mg. dry powder in 30 min. were given as: 146 mm.<sup>3</sup> from cabbage, 62 mm.<sup>3</sup> from spinach and 41 mm.<sup>3</sup> from carrots. For direct comparison with these figures, 30 min. readings adjusted for 1 g. fresh weight are included in Table I.

TABLE I  
*Distribution of Glutamic Acid Decarboxylase in Various Plants*

Material	$\text{QCO}_2$	mm. <sup>3</sup> $\text{CO}_2$ in 30 min./g. fresh tissue
Yellow Squash	1.2 -3.0	350-860
Italian Squash	2.59	845
White Squash	1.9 -2.8	510-780
Avocado	1.2 -1.7	455-535
Avocado Seed	0.07	12
Green Pepper	1.54	494
Hot Pepper	0.97	292
Cucumber	0.87	260
Radishes	0.85	300
Parsley (tops)	0.81	274
Carrots	0.68	222
Asparagus tips	0.63	223
Parsnips	0.62	180
Celery (tops)	0.60	189
Green Peas	0.50	149
Eggplant	0.35	108
Turnips	0.33	120
String Beans	0.31	102
Artichoke	0.29	95
Vegetable Pear (Chayote)	0.29	77
Lettuce	0.21	64
Spinach	0.20	58
Mustard Greens	0.16	45
Cabbage, green leaves	0.15-0.31	31- 93
Cabbage, white leaves	0.08	13- 15
Beets	0.15	48
Ground Artichoke	0.13	29
Tomatoes	0.12	21
Corn	0.12	25
Okra	0.09	23
Horseradish	0.08	28
Rutabaga	0.03-0.08	8- 13
Broccoli	0.06	8
Sweet Potatoes	0.05	8
Cauliflower	0.05	4
Sunflower Seeds	0.03	8

### 3. Preparation of Clear Enzyme Solutions

When suspensions, prepared as described in the previous section, were centrifuged, opalescent supernatant solutions were obtained which showed a considerable portion of the enzymatic activity of the original suspension. As the activity of such extracts might conceivably be due to the finely dispersed particles causing the residual turbidity, attempts were made to remove this turbidity without loss of glutamic acid decarboxylase. Filtration through Seitz filters yielded clear solutions without enzymatic activity. Treatment with 0.5% Norit also removed glutamic acid decarboxylase completely, whereas 0.1% Norit adsorbed about 40–50% of the decarboxylase but failed to remove turbidity completely. A variety of other adsorbing agents was tried and some of the results obtained are listed in Table II.

TABLE II  
*Effect of Adsorbing Agents and Filtration on Turbidity and  
Enzymatic Activity of Carrot Extracts*

A. Extract 43. Fresh Carrots. $Q_{CO_2}=0.22$					
Treatment of Extract					
	None	Kaolin 0.25%	Permutit 0.25%	$Al_2O_3$ 0.25%	Filtration 36060-F
"Turbidity"	0.407	0.041	0.046	0.027	0.017
Turbidity in % of value for starting material	100	10.1	11.3	6.6	4.2
Activity in % of value for start- ing material	100	67	82	86	87
B. Extract 19, Old Carrots. $Q_{CO_2}=0.56$					
Treatment of Extract					
	None	Kaolin 0.50%	Permutit 0.50%	$Al_2O_3$ 0.50%	Filtration 36060-F
"Turbidity"	1.044	0.286	0.361	0.496	0.018
Turbidity in % of value for starting material	100	27.4	34.6	47.5	1.7
Activity in % of value for start- ing material	100	92	100	86	73

\* Standardized according to Brockmann.

Turbidities were measured with a Lumetron Photoelectric Colorimeter, Model 402-E using 10 mm. cuvettes and filter M-575. Two readings were taken, one with the cuvette as close as possible to the photoelectric cell and one as far away as possible. Distilled water shows only a very slight difference between the readings with the cuvette in both positions. When the same experiment was performed with turbid extracts, considerably less light reached the photocell from the distant position and this loss of light due to scattering was used as a numerical value for the degree of turbidity present. The advantage of this method is that changes in light absorption caused by the removal of pigments by adsorbing agents are automatically compensated and do not confuse the results. An example may serve to illustrate this procedure: A cuvette filled with turbid extract was placed next to the photocell and the instrument was adjusted to read 100% transmission. The cuvette was then moved away from the cell to the other end of the compartment and a reading showed 38.9% transmission, corresponding to an extinction of 0.410. A cuvette with distilled water, manipulated the same way, showed a reading of 99.4% in the distant position, corresponding to an extinction of 0.003. This reading for the "blank" was subtracted from 0.410 and gave 0.407 as a measure of the turbidity of the extract. The same extract diluted 1:1 with water gave a "turbidity-value" of 0.226. A second extract, with a "turbidity" of 1.044 gave a value of 0.115 after dilution with water 1:10. These data show that there is a reasonable proportionality between the degree of turbidity and the numerical values obtained.

Clear extracts were finally prepared without adsorbing agents when it was found that the use of fritted glass filters would lead to clear solutions. The method for the preparation of extracts for all further experiments was as follows: 100 g. carrots was mixed in a Waring Blender for 2-3 min. with 100 ml. ice cold *m*/15 phosphate buffer pH 6.4 and the suspension was stored at 4°C. for one hour. The mixture was then filtered through cheese cloth and the filtrate centrifuged for 15 min. (Size 2 centrifuge, conical head, 4000 r.p.m.). The turbid and light orange supernatant fluid was filtered through a Corning fritted glass filter No. 36060-600-M and subsequently through filter No. 36060-600-F. This procedure yielded water-clear extracts, provided the filtration through the second, fine porosity filter was carried out with only very slight suction. When the full vacuum obtainable with a water suction pump was applied, the filtrates were clear only until about 1/3 of the total volume had been filtered. By this time fine particles had usually found their way through the fritted glass and the filtrate became turbid. A comparison between the results for Extract 43 and Extract 19 in Table II shows that the filtration method using slight suction regularly produced clear extracts, independent of the degree of turbidity of the starting material. The effectiveness of adsorbing agents, on the other hand, varied considerably from one extract to the next.

From 100 g. carrots between 110 and 140 ml. filtrate, pH about 6.4, was obtained. For most experiments an adjustment to about pH 5.7 was made by adding 18 ml. *m*/15  $\text{KH}_2\text{PO}_4$  solution and 2 ml. *m*/15  $\text{H}_3\text{PO}_4$  to 20 ml. filtrate. The enzymatic activity of clear carrot extracts ranged between 25 and 70% of the activity of the corresponding suspensions. The enzyme was found to be more soluble when old car-

rots were used and its solubility from fresh carrots increased during storage at 4°C., probably as a result of autolytic processes. A suspension of fresh carrots with a  $Q_{CO_2}$  value of 0.32 for example, gave an extract with a  $Q_{CO_2}$  of 0.08. Pieces of the same carrots were stored in the refrigerator for 4 days and the  $Q_{CO_2}$  value of an extract prepared after that period of time was found to be 0.21, i.e., the solubility increased from 25 to 66%. Table III illustrates the course of an experi-

TABLE III  
*Comparison between Suspension and Extract from Fresh Carrots*

Min.	mm % $CO_2$ liberated at pH 5.9 and 37°C		
	Suspension 0.9 g carrots	Suspension 0.54 g carrots	Extract from 0.9 g carrots
10	43	26	19
20	69	41	34
30	88	53	42
40	100	59	51
50	111	66	54
60	117	67	57
80	129	76	63
100	140	85	67
120	140	85	72
$Q_{CO_2}^{N_2}$	0.287	0.289	0.127

ment in which the activity of a suspension is compared with that of a clear extract, containing 44% of the total activity. From a comparison between the activities of suspensions containing 0.9 g. and 0.54 g. of carrots in the same table it can be seen that there is good proportionality between the amount of suspended material and the volumes of  $CO_2$  formed.

#### 4. Effect of Storage and Dialysis

Whole carrots, when stored for several weeks at 4°C., showed no loss in glutamic acid decarboxylase content. Carrot extracts, however, retained constant activity only for about 24 hrs. and then gradually became less active with a loss of about 25% in 3 days. The behavior of glutamic acid decarboxylase in this respect resembled that of di-oxyphenylalanine decarboxylase from pig kidneys (4), in which case

no change in activity was observed on storage of whole kidneys for one week, whereas extracts lost about one-half their activity within 2 days.

Glutamic acid decarboxylase from carrots lost activity much faster, as compared to the rate of inactivation on mere storage, when its solutions were dialyzed against *m*/15 phosphate buffer pH 6.4 at 4°C. (see Table IV). These observations were interpreted as probably being due to removal of a prosthetic group. The activity of extracts sub-

TABLE IV  
*Effect of Storage and Dialysis on Activity of Carrot Extracts*

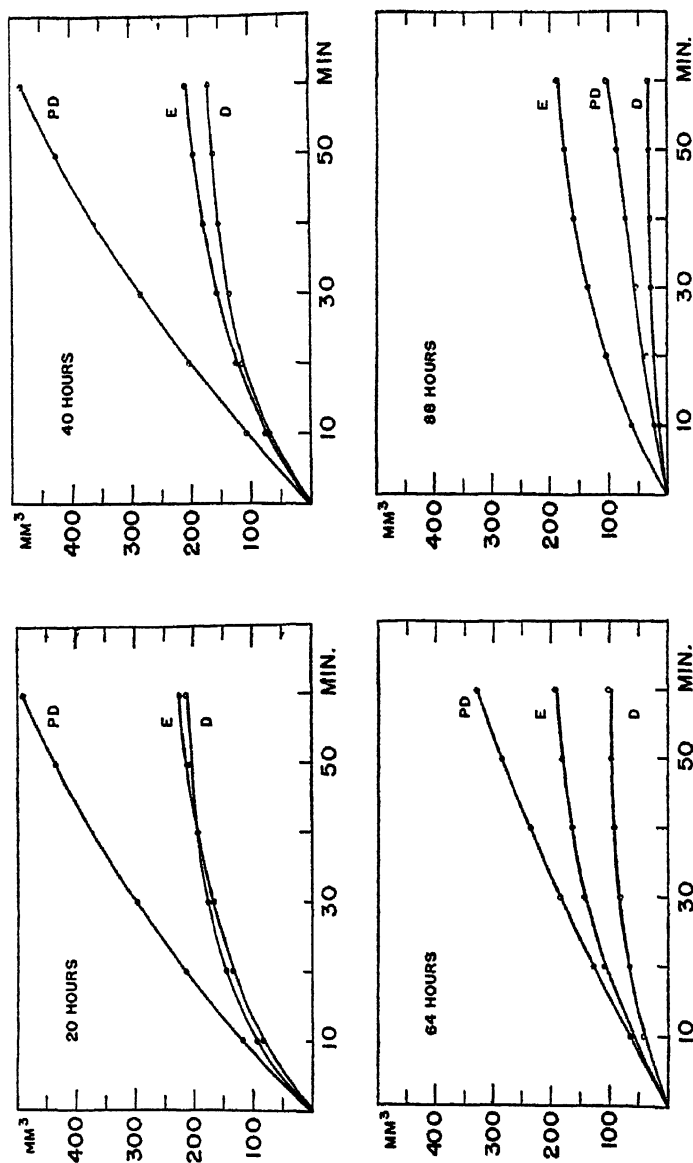
mm.<sup>3</sup> CO<sub>2</sub> liberated at pH 5.9 and 37°C.

min.	Fresh extract	Stored 18 hrs.	Dialyzed 18 hrs.
10	29	29	17
20	50	48	24
30	66	65	31
40	76	72	34
50	85	78	35
60	91	82	38
80	100	86	41
100	105	92	43
120	108	94	44

TABLE V  
*Effect of Pyridoxal on the Activity of Dialyzed Carrot Extract*

Min.	mm. <sup>3</sup> CO <sub>2</sub> liberated at pH 5.9 and 37°C.		
	Stored 18 hrs.	Dialyzed 18 hrs.	Dialyzed 18 hrs. 5 mg. pyridoxal added
10	37	29	39
20	64	63	74
30	84	78	91
40	100	91	103
50	110	98	113
60	116	103	118
80	125	111	124
100	131	117	132
120	134	123	138
Q <sub>CO<sub>2</sub></sub> <sup>N<sub>2</sub></sup>	0.32	0.25	0.33

FIG. 1  
Inactivation of Glutamic Acid Decarboxylase in Carot Extract by Dialysis and Restoration  
of Activity on Addition of Pyridoxal Phosphate



Explanation of Symbols:

E—Stored extract  
D—Dialyzed extract

PD—Dialyzed extract, pyridoxal phosphate added  
All curves are corrected for volume changes during dialysis

jected to dialysis was restored by incubation with pyridoxal, as shown in Table V. A much more pronounced activity, even considerably beyond that of the original extract, occurred, however, when small amounts of "phosphorylated" pyridoxal<sup>\*</sup> instead of pyridoxal were added. Fig. 1 shows the slow inactivation of an extract during storage, the progressive loss of activity on dialysis and the restoration of high activity on addition of an amount of pyridoxal phosphate, equivalent to 40  $\gamma$  of its barium salt in each experiment. During the course of dialysis a point was finally reached at which irreversible changes took place, most likely on the protein moiety of the enzyme molecule. After 88 hrs. of dialysis the addition of pyridoxal phosphate still resulted in an activation but the activity of the stored extract was not reached any more.

### DISCUSSION

The finding that clear solutions of glutamic acid decarboxylase from plants can be obtained with ease makes it difficult to explain Okunuki's contrary results. It is possible that Okunuki did most of his solubility experiments with pollen of *Lilium auratum* and that the enzyme from this source is much less soluble than from other material. The comparatively low activity assigned by Okunuki to carrots may very well be due to losses during the removal of juice by means of a hand press. Carrots have a water content of 83–91% (5) so that 100 mg. dry powder used by Okunuki is equivalent to 600–1100 mg. of fresh carrots. The formation of 41 mm.<sup>3</sup> CO<sub>2</sub> by 100 mg. dry powder in 30 min., which corresponds to about 20 mm.<sup>3</sup> in 10 min. (see Table III), would therefore result in a  $Q_{CO_2}$  of 0.1–0.2, which is of the magnitude expected for the amount of enzyme left behind during extraction.

The ease with which the plant enzyme loses its prosthetic group on dialysis is in contrast to observations by Taylor and Gale (6) with bacterial glutamic acid decarboxylase from coliform organisms, where a resolution could not be accomplished, suggesting to these authors the absence of codecarboxylase. A resolution of the bacterial enzyme (from *E. coli* No. 4157 A.T.C.C.) was obtained, however, on dialysis by Umbreit and Gunsalus (7), who showed that full activity could be restored on incubation with pyridoxal and adenosine triphosphate, apparently with an enzymatic phosphorylation of pyridoxal as an

<sup>\*</sup> Obtained through the courtesy of Dr. W. W. Umbreit.



intermediate reaction. The exact structure of the prosthetic group of bacterial amino acid decarboxylases has not as yet been definitely proved. Gale and Epps (8) reported that purified "codecarboxylase" from yeast did not contain phosphorus, but Baddiley and Gale (9) confirmed the effectiveness of pyridoxal phosphate as activator for several bacterial apodecarboxylases and found 0.8–1.0% P in codecarboxylase from yeast.

The possibility that the treatment of pyridoxal with phosphorylating agents (10), which eliminates the need for ATP, might produce, not a pyridoxal phosphate but a phosphorus-free pyridoxal derivative, has been mentioned by Umbreit, Bellamy and Gunsalus (10) to reconcile the earlier discrepancies. It has recently been shown, however, (11), that an actual phosphorylation of pyridoxal takes place by treatment with  $\text{POCl}_3$ , resulting, one is inclined to assume (12), in the formation of a phosphoric acid ester of pyridoxal by condensation with the hydroxymethyl group in position 5. The synthetic product contained (as Ba-salt) 6.2% P, *i.e.* 1 atom of phosphorus/mol of pyridoxal (11).

Regardless of what the final structure of "pyridoxal phosphate" will turn out to be, the experiments reported in this paper show that an amino acid decarboxylase from higher plants is activated by the same pyridoxal derivative which was discovered by Gunsalus *et al.* (10, 12) to be necessary for activation of the bacterial enzymes. The results obtained with higher plants are, therefore, additional evidence for the importance of vitamin  $\text{B}_6$  derivatives in the protein metabolism of living tissues.

#### SUMMARY

1. The distribution of glutamic acid decarboxylase in a variety of higher plants has been investigated. Squash, avocado and green pepper were found to be particularly rich sources of this enzyme.

2. A method is described for the preparation of clear solutions of glutamic acid decarboxylase from carrots.

3. The resolution of this enzyme into protein carrier and prosthetic group is accomplished by dialysis at pH 6.4.

4. The addition of pyridoxal and particularly of small amounts of "pyridoxal phosphate" (a pyridoxal derivative obtained by treatment of pyridoxal with  $\text{POCl}_3$ ) to solutions of the apoenzyme restores enzymatic activity. This finding suggests that "pyridoxal phosphate" is the prosthetic group of glutamic acid decarboxylase in higher plants.

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# Physical and Chemical Studies on Southern Bean Mosaic Virus

## I. Size, Shape, Hydration and Elementary Composition

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### INTRODUCTION

Application of the newer physical methods to the study of viruses has yielded a large amount of information about these infectious agents. Sufficient physical measurements have now been obtained for tobacco mosaic (1, 2), tomato bushy stunt (3, 4, 5), vaccinia (6, 7, 8), rabbit papilloma (9, 10) and influenza (11, 12) viruses to provide fairly reliable pictures of their ultimate particles.

Recent isolation of southern bean mosaic virus (13, 14) afforded an opportunity for applying some of the same procedures to obtain a physical picture of its particles. Its size was determined, on an anhydrous basis, by sedimentation, viscosity, diffusion and pycnometric partial specific volume measurements. The question of hydration was afterwards answered by sedimenting the virus in solvent media of varying densities. It was then possible to deduce the shape of the particles and calculate their size on a hydrated basis.

Additional information about the virus was obtained by analyzing it for elementary composition.

### EXPERIMENTAL

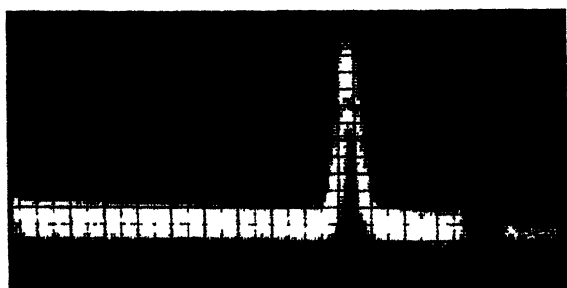
Except when otherwise indicated, all studies were carried out with noncrystalline virus preparations, obtained by centrifugal and chemical methods as described elsewhere (14).

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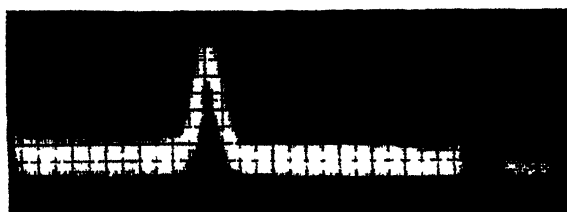
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*Sedimentation Studies.* Sedimentation measurements were made in 0.1 *M* potassium phosphate buffer at pH 7.1, in a Bauer-Pickels analytical centrifuge (15) equipped with a Svensson optical system (16). Sedimentation diagrams, shown in Fig. 1,



40 min.



80 min.



120 min.

FIG. 1

Svensson Sedimentation Diagrams of Southern Bean Mosaic Virus  
Virus concentration, 0.5 g./100 ml.; centrifuge speed, 14,800 R.P.M.;  
angle of inclined slit, 60°

indicated the presence of only a single component possessing a high degree of homogeneity. The sedimentation constant at a virus concentration of 0.5 g./100 ml. was 114 S, corrected to water at 20°C.

The relationship between virus concentration and the reciprocal of the sedimentation rate is shown graphically in Fig. 2, from which it is seen that similar results were obtained with chemically isolated and centrifugally isolated virus preparations. A

straight line was fitted to the data by the method of least squares. The sedimentation rate obtained by extrapolation to zero concentration was 115 *S*.

*Viscosity Studies.* Viscosity measurements were made in 0.1 *M* potassium phosphate buffer at pH 7.1 in an Ostwald viscometer. One sample of virus obtained by high speed centrifugation and two samples representing pooled mixtures of chemically and centrifugally isolated virus preparations were used. The results are shown in Fig. 3. As will be seen, the data indicated a straight line relationship between specific viscosity and virus concentration.

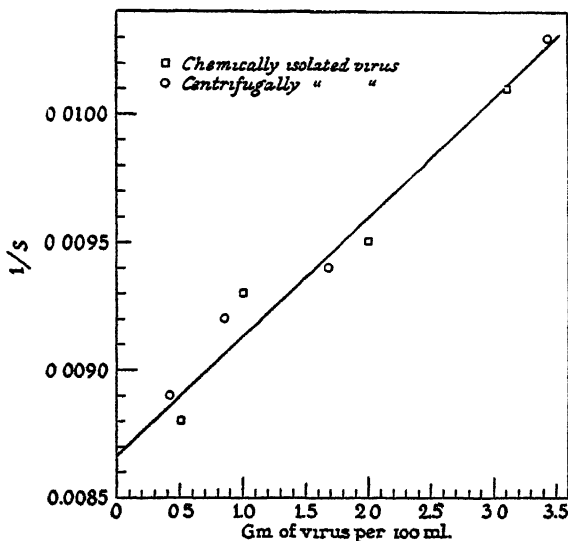


FIG. 2

Relationship between Virus Concentration and Reciprocal of Sedimentation Rate  
The sedimentation rates are corrected to 20°C.

It has been shown by Laufer (17), for tobacco mosaic virus, that if the sedimentation data are corrected for viscosity of the solution, instead of for that of the solvent, the sedimentation constant is independent of concentration. When the same sort of corrections were made for the present data, the sedimentation constant of southern bean mosaic virus was likewise found to be independent of concentration.

*Diffusion Studies.* Diffusion analyses were made at three different virus concentrations in 0.1 *M* potassium phosphate buffer at pH 7.1 at 0.3°C. in the electrophoresis cell as used by Longworth (18). All values were corrected to 20°C. with water as the solvent. Figures shown in Table I for the diffusion constant, calculated by two different methods, agreed well. Any effect which concentration of virus may have had on diffusion rate was apparently too slight to exceed experimental error over the concentration range studied. The final average,  $1.34 \times 10^{-7}$  sq. cm./sec., was, therefore, calculated. Theoretical concentration gradient curves were congruent with the observed ones, indicating that free diffusion took place.

Diffusion constant measurements determined from sedimentation diagrams provided a more sensitive test (19) for homogeneity. Data from the sedimentation experiment illustrated by Fig. 1 were used. Tracings of the top and the bottom edges of the Svensson curves differed seriously in earlier exposures but agreed more closely in the later ones. By the maximum height-area method, agreement between results calculated from the upper and lower tracings were fairly good for the 100- to 120-minute photographs. The average diffusion constant was  $1.23 \times 10^{-7}$  sq. cm./sec.,

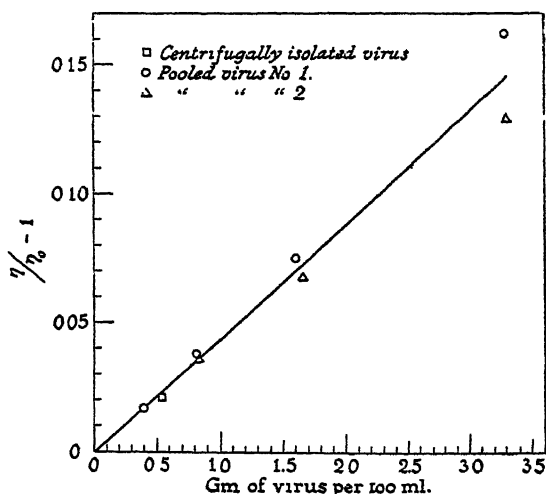


FIG. 3

Relationship between Virus Concentration and Specific Viscosity

TABLE I

*Diffusion Measurements on Southern Bean Mosaic Virus*

Virus preparation no.	Virus concentration	Diffusion constant	
		Maximum height-area method	Inflection point method
	$\mu./100$ ml.	$\text{sq. cm./sec.} \times 10^7$	$\text{sq. cm./sec.} \times 10^7$
1	0.50	1.40	1.30
1	1.12	1.35	1.42
2	0.74	1.20	1.26
Average $D_{20}$ .....		1.35	1.33
Final average $D_{20}$ .....			1.34

## SOUTHERN BEAN MOSAIC VIRUS

corrected to water at 20°C. Had the virus been inhomogeneous with respect to size, the sedimentation diagrams should have yielded abnormally high diffusion constants. For example, a standard deviation in sedimentation rate of 2% due to differences in particle sizes would have given an apparent diffusion constant of about  $2.9 \times 10^{-7}$  under the sedimentation conditions used. Thus the actual value obtained in the present work, though probably subject to certain optical errors, is contrary to an interpretation of inhomogeneity.

*Partial Specific Volume Studies.* Measurements of partial specific volume on a dry basis were carried out pycnometrically in distilled water. Amounts of solids present in each test were determined by transferring aliquots to weighed porcelain ignition boats, evaporating to dryness in a desiccator at room temperature and finally drying to a constant weight *in vacuo* at 100°C. A measurement on noncrystalline virus at 8.20 g./100 ml. in a 25 ml. pycnometer at 26°C. yielded a value for the partial specific volume of 0.687 ml./g. Twice crystallized material, at 3.10 g./100 ml. in a 10 ml. pycnometer at 30°C., gave a value of 0.705 ml./g. In the latter determination, the distilled water against which the sample was first dialyzed was made weakly alkaline with NaOH to prevent crystallization of the virus (20). Since both the noncrystalline and crystalline virus preparations used were completely homogeneous when tested by moving boundary electrophoretic analysis (21) and, furthermore, as will be shown later, revealed no appreciable differences in elementary composition, the average value of partial specific volume was calculated, namely, 0.696 ml./g.

*Hydration Studies.* Measurements of partial specific volume on a hydrated basis were made by the method of sedimentation in solvents of varying densities (22). Solutions of sucrose in an electrolyte medium of 0.1 *M* potassium phosphate buffer at pH 7.1 were used for this purpose. Values for the densities of sucrose solutions were obtained from standard tables, and viscosity values were obtained by direct measurements at 25.8°C. combined with interpolation of data by Bingham and Jackson (23).

In Fig. 4 the sedimentation rates of the virus, corrected to water at 20°C. for all factors except the density due to the sucrose, are plotted against the densities of the medium. At a concentration of 50% sucrose, where the density was equal to 1.230, no sedimentation occurred. Thus, the density of the particles under these conditions also was 1.230, corresponding to a partial specific volume of 0.813 ml./g.

As pointed out by other investigators employing this method (7, 11, 12), the curvature of lines such as shown in Fig. 4 indicates either a dehydrating action of the medium at high concentrations or an actual combination of the molecules of the medium with the sedimenting particles, or both. This effect can be corrected to a certain extent by the construction of tangents to the curves at points of zero concentration of suspending medium. The present data were, therefore, fitted to an equation of the general form,  $y = a + bx + cx^2$ , namely,  $S = 862.1 - 955.5 d + 207.4 d^2$ , where  $S$  represents the sedimentation rate of the virus and  $d$  the density of the medium. The slope of the



tangent at the point of zero concentration of the suspending medium was calculated by differentiating the given equation and substituting for  $d$  the density of water at 20°C. The tangent obtained, shown by the dotted line in Fig. 4, intercepted the ordinate of zero sedimentation at a density value of 1.21, corresponding to a partial specific volume of 0.827. The small degree of departure of the tangent from the experimental curve suggested that the dehydrating or combining actions of the sucrose medium on the virus particles were slight.

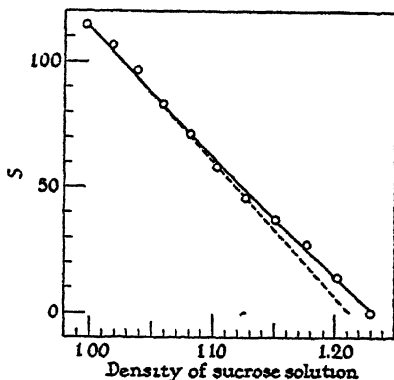


FIG. 4

Relationship between Sedimentation Rate of Virus and Density of Sucrose Medium

Circular points represent experimental results. The continuous line was drawn from an equation to which the data were fitted. The dotted line is the tangent to the curve at the point of zero concentration of the suspending medium.

*Calculations of Size, Shape and Degree of Hydration.* The particle weight of southern bean mosaic virus on an anhydrous basis was calculated by means of the Svedberg equation (24, eq. 3a)

$$M = \frac{RTs}{D(1 - V_p)}$$

in which  $M$  is the particle weight;  $R$ , the gas constant;  $T$ , the absolute temperature;  $s$ , the sedimentation constant;  $D$ , the diffusion constant;  $V_p$ , the partial specific volume; and  $\rho$ , the density of the solvent. For substitution in this equation,  $s$  was taken as 115, the sedimentation constant at zero concentration. Since the value of the diffusion constant at zero concentration was not available, it was estimated as follows on the basis of a viscosity correction assumed to apply to diffusion as well

as to sedimentation (17): the value of the diffusion constant,  $1.34 \times 10^{-7}$ , at the average concentration tested, 0.8 g./100 ml., was multiplied by the relative viscosity of the solution, 1.035, obtained from Fig. 3, to give the theoretical value,  $1.39 \times 10^{-7}$ , at infinite dilution. Substitution of these values in the above equation gave a value of 6.63 millions for the particle weight of the virus.

The same corrected values were used, as recommended by Oncley (25), for the calculation of the ratio of the observed molecular frictional constant,  $f$ , to that of a spherical particle of the same weight,  $f_0$ , according to the equation (24, eq. 70b),

$$f/f_0 = 10^{-3} \left( \frac{1 - V_p}{D_{20}^2 S_{20} V} \right)^{1/3}$$

The frictional ratio thus calculated was 1.25. For anhydrous particles, this value corresponded to an axial ratio,  $a/b$ , of 5 to 1, based on the equation of Perrin for oblong ellipsoids (24, eq. 71),

$$f/f_0 = \frac{\sqrt{1 - (b/a)^2}}{(b/a)^{2/3} \ln \frac{1 + \sqrt{1 - (b/a)^2}}{(b/a)}}$$

where  $a$  is the major and  $b$  is the minor axis. For spherically shaped particles, on the other hand, the frictional ratio indicated the combination of 0.67 g. of water with each g. of anhydrous virus, calculated from the equation of Kraemer (24, eq. 125),

$$f/f_0 = \left( \frac{rV_2 + V_1}{V_1} \right)^{1/3}$$

where  $r$  represents the number of grams of component 2 combined with 1 g. of component 1, and  $V_2$  and  $V_1$  represent the partial specific volumes of components 2 and 1, respectively. If the value for the diffusion constant was not corrected for viscosity as indicated above, the calculated axial ratio was 5.5 to 1 and the hydration, 0.78 g. of water/g. of virus.

The intrinsic viscosity of the virus,  $[\eta]$ , which is defined as the limiting value at infinite dilution of the specific viscosity divided by the volume fraction of the virus, was calculated from the data of Fig. 3 to be 6.35. For anhydrous particles, this value indicated an axial ratio of 5.5 to 1 based on Simha's equation (26, eq. 6a),

$$[\eta] = \frac{(a/b)^2}{15 (\ln 2a/b - 3/2)} + \frac{(a/b)^2}{5 (\ln 2a/b - 1/2)} + \frac{14}{15}$$

where the symbols have the significance indicated in previous equations. Alternatively, however, for spherically shaped particles, the viscosity data pointed to a combination of 1.07 g. of water/g. of anhydrous virus, calculated by means of Einstein's equation (27),

$$\eta/\eta_0 - 1 = 2.5 \phi,$$

where  $\phi$  represents the volume fraction of the solute.

The value, 0.827, for the partial specific volume of the hydrated virus particles based on the tangent to the curve of Fig. 4 showing the relationship of sedimentation rate to density of sucrose medium, taken in conjunction with the value, 0.696, determined pycnometrically for the anhydrous particles, indicated a combination of 0.76 g. of water with each g. of anhydrous virus, calculated by means of the equation of Lansing and Kraemer (24, eq. 117),

$$(r + 1)V_{12} = rV_2 + V_1$$

where  $r$ ,  $V_1$  and  $V_2$  have the meanings already indicated, and  $V_{12}$  represents the partial specific volume of the component formed by the combination of components 1 and 2. This value for the hydration of spherical particles falls between those calculated from the frictional ratio on the one hand and from viscosity measurements on the other. The choice of hydrated spherical particles as an interpretation of the data thus appeared to be correct.

In all of the above calculations of hydration it was assumed that the density of water bound to the virus particles was the same as that of free water, and that the anhydrous partial specific volume of the virus was essentially constant over the temperature range 20–30°C. Since each method of determining hydration may be subject to theoretical as well as experimental error, an average figure of hydration of 0.83 g. of water/g. of anhydrous virus was calculated. On this basis, the hydrated, spherical virus units possessed a particle weight of 11.6 millions and a diameter of 312 Å. If uniform shrinkage occurred during drying, the dehydrated particles would have a diameter of 244 Å.

The correctness of the above picture of southern bean mosaic virus is further corroborated by the fact that electron micrographs of purified preparations of the virus also indicate the ultimate units to be spherical in shape (14, 28).

*Elementary Analysis.* Analyses of southern bean mosaic virus for elementary composition are shown in Table II. All measurements, except the Kjeldahl nitrogen

TABLE II  
*Elementary Analysis of Southern Bean Mosaic Virus*

Virus preparation	C	H	N		P		S	Ash
			Dumas	Kjeldahl	Pregl	King		
I. Uncrystallized	45.15	6.51	16.74	16.8	1.76		1.13	6.38
II. Crystallized from water	45.96	6.66	17.33		1.87		1.35	5.20
III. Crystallized from acetate	45.82	6.44	17.21	16.8	2.00	1.92	2.17, 0.71	5.00
Average	45.64	6.54	17.0		1.89		1.34	5.68

and the King (29) phosphorus determinations, were made by Dr. Adalbert Elek. The samples before analysis were shown to be homogeneous by electrophoretic tests (21). The agreement in values for the different preparations was good except for sulfur for which reproducible results were not obtained. Preparations I and III, corresponding to the noncrystallized and crystallized virus samples employed earlier in the pycnometric measurements of partial specific volume, do not reveal significant differences in composition.

## DISCUSSION

Compared in size with other presumably spherical plant viruses, southern bean mosaic virus with an anhydrous particle weight of 6.63 millions is smaller than bushy stunt virus, 7.6 to 10.6 millions (3, 4, 5), possibly about the same size as Princeton, Potato and Tobacco VI strains of tobacco necrosis virus, based on similarity in sedimentation rates (30, 31, 32), and larger than tobacco ringspot virus, 3.4 millions (33), alfalfa mosaic virus, 2.1 millions (34), and the Rothamsted strains of tobacco necrosis virus, 1.6 to 1.85 millions (35, 36, 37). It is smaller in size than the rod-shaped tobacco mosaic virus, a representative strain of which has a particle weight of 31.6 millions (2).

The partial specific volume of southern bean mosaic virus, as well as that of other viruses, particularly the spherical ones, is lower than that of the more common smaller proteins (24). This may be related to the nucleic acid content of viruses since nucleic acid isolated from tobacco mosaic virus has been shown by Cohen and Stanley (38) to have a low

partial specific volume of 0.578. As suggested by Pirie and coworkers (30), the nucleic acid content, by virtue of the phosphate radicals, may also be related to the high ash content.

The nitrogen content of southern bean mosaic virus appeared higher than that of other viruses. The sulfur and phosphorus contents, very similar to those of strains of tobacco necrosis virus (30, 32, 35), also were relatively high, the phosphorus value, 1.9%, being exceeded only by that of tobacco ringspot virus, namely, 3.2–4.1% (33).

### SUMMARY

Southern bean mosaic virus has been shown by sedimentation, viscosity, diffusion and pycnometric partial specific volume measurements to have an anhydrous particle weight of 6.63 millions. The ratio of the observed molecular frictional constant to that of a spherical particle of the same weight was 1.25, corresponding to anhydrous particles with an axial ratio of 5 to 1, or, alternatively, to spherical particles hydrated to the extent of 0.67 g. of water/g. of anhydrous virus. Viscosity data indicated similarly either an axial ratio of 5.5 to 1 for anhydrous particles or a hydration of 1.07 g. of water/g. of virus for spherical particles. Sedimentation measurements in sucrose media of varying densities indicated a hydration of 0.76 g. of water/g. of virus and thus established unambiguously the hydrated spherical nature of the virus unit. From the average value of hydration obtained by the three methods, a weight of 11.6 millions and a diameter of 312 Å were calculated for the virus in the hydrated state. On the assumption that uniform shrinkage occurred during drying, the dehydrated particles were calculated to have a diameter of 244 Å.

The elementary composition of the virus was found to be 45.64% C, 6.54% H, 17.0% N, 1.89% P, 1.34% S, and 5.68% ash.

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## Pectic Enzymes VII. The Preparation of Tomato Pectin-Methylesterase \*

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### INTRODUCTION

The methyl ester groups of pectin may be removed by the action of acids, alkali or the enzyme pectin-methylesterase (PM). Partially demethylated or "low methoxyl" pectinic acids have recently attracted considerable attention because of their ability to form gels with less sugar than is required for pectin. PM is therefore a useful aid in preparing and studying low ester pectins, as well as a tool in studying the structural complexities of pectin itself.

PM is widely distributed in the plant kingdom. However, there are few sources which contain it in abundance. Alfalfa, tobacco and lilac leaves (1), and egg plant (2), for example, have been shown to contain moderate PM activities. Kertesz (3, 4) has shown that tomato fruit is relatively rich in the enzyme and, more recently, MacDonnell, Jansen and Lineweaver (5) have demonstrated that orange flavedo and albedo are also good sources. Dry preparations of the enzyme, such as might be useful for commercial and experimental work, have been prepared from clover (6), tomato fruit (2) and orange flavedo (5). Kertesz (4), and Willaman and Hills (2) have shown that the PM in comminuted tomatoes is largely adsorbed on the pulp. The latter authors "desorbed" PM by raising the pH of the juice to between 5 and 7. By filtering such a mixture, the eluted enzyme could be obtained in the filtrate. They also prepared a powdered form of the enzyme by squeezing the serum from comminuted tomatoes through a suitable filter cloth and dehydrating the resulting pulp in acetone and air. This product contained 86 PMU/g.<sup>2</sup> MacDonnell, Jansen and

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<sup>2</sup> The term PMU refers to pectin methylesterase units as defined by Kertesz (3). In our work slight modifications of the original procedure have been introduced, in



Lineweaver (5) extracted orange flavedo with borate-acetate buffer at pH 8.2 and precipitated the enzyme from the extract by "0.6 saturation" with ammonium sulphate. The precipitate dried in vacuum at 5°C. contained 1,395 PMU/g.<sup>3</sup>

It is the purpose of this paper to report methods by which concentrated solutions and dry preparations containing 200,000 PMU/g. may be obtained from tomatoes.

### EXPERIMENTAL

The observation was recently made in this laboratory that a small precipitate was formed when tomato extract made by the method of Willaman and Hills (2) from the frozen comminuted fruit was dialyzed against distilled water until  $\text{Cl}^-$  free. Tests showed that less than 1% of the original PM activity remained in the filtrate and presumably the remaining activity was in the precipitate. The precipitate was separated by centrifuging and extracted by standing a short time with 10% NaCl solution. When the undissolved material (which included red pigments) was centrifuged off, the resulting extract contained about 50% of the PMU theoretically present in the precipitate. Dialysis of this extract resulted in another precipitate. The supernatant liquid contained less than 5% of the activity which had been present. The precipitate was again separated and extracted with a smaller volume of salt solution. Although much of the solid material remained undissolved, 98% of the theoretical PM activity was found in the extract after the solids were centrifuged off. Repetition of these experiments showed that, after the large initial loss, practically no further losses occurred in subsequent dialysis-salt extraction cycles and that, by diminishing the volume of salt solution for succeeding extractions, the

that a Beckman pH meter was used to follow pH changes and the determinations were made at pH 7.0 instead of 6.2. The substrate was a 0.5% solution of 190 grade California Fruit Grower's Exchange citrus pectin with NaCl added to a concentration of 0.1 *M*. The reaction temperature was 23°C. The activities obtained are slightly higher than in the original method.

<sup>3</sup> Lineweaver and Ballou (7) have recently suggested a different expression for PM activity, which they name pectinesterase unit [PE. u.], and state that one [PE. u.] is equivalent to 930 PMU. Although conditions chosen for the [PE. u.] estimation vary slightly from those used in this work for PMU determinations, salt concentration and pH optimum curves (7) and experience in this laboratory show that the activities obtained by the two methods at equal temperatures are nearly identical. In this paper we have quoted some data of earlier investigators, converting the PM activities to PMU for convenient comparison.

purity and concentration of the enzyme could be greatly increased. A very small quantity of a solution containing 176,000 PMU/g. of dry matter was actually obtained in this way.

This would seem to be a useful method for concentrating and purifying the enzyme, but on a large scale it would be impractical because of the great volume of material which would have to be dialyzed. Consequently, two supplementary procedures were developed for reducing the volume of large quantities of juice before applying dialysis.

In the first procedure, the enzyme is desorbed, then both the suspended solids and water are removed by freeze concentration while dissolved materials including the PM, sugars and organic acids, *etc.*, remain in the concentrate. In the second procedure the suspended particles, containing about 95% of the enzyme, are separated from the serum by centrifuging or filtration and the PM then eluted in a small quantity of salt solution.

#### *Procedure I*

Approximately two bushels of ripe tomatoes were comminuted in a stainless steel hammermill. Sufficient 5 *N* NaOH was then added to raise the pH to about 8. After standing for 24 hours at 1°C., the skin debris and seeds were removed in a stainless steel basket centrifuge using a cloth filter. The turbid, red filtrate was slowly frozen at about - 11°C. in large aluminum containers. The frozen mass was removed from the containers and disintegrated in the hammermill at 1°C. Because of the high melting point of this low-solids juice, it was possible to separate the liquor from the ice in the basket centrifuge<sup>4</sup> at room temperature. In fact the ice remained too cold (- 6°C.) for best separation and retained considerable PM activity. The raw ice before concentration contained a total of 676,000 PMU; the ice remaining after concentrating contained 205,800 PMU; and the concentrated liquor contained 471,500 PMU. The filtrate was refrozen at - 22°C. As the remaining ice still contained about 30% of the total activity, it was allowed to come to - 2°C. and re-centrifuged at room temperature. The resulting filtrate was more dilute than the first, but over 2/3 of the enzyme content of the ice was recovered. This filtrate was also frozen at - 22°C. Each frozen filtrate was again concentrated by comminuting in the hammermill and centrifuging, and the filtrates combined. The total PMU present in the combined filtrates was about 600,000. The concentration of enzyme was 825 PMU/g. of dry matter or 278 PMU/mg. of total nitrogen.

An amount of combined filtrates (3,500 ml.) containing about 325,000 PMU was dialyzed in large viscose sausage casings for three days against distilled water. The precipitate which formed was allowed to settle and separated from the supernatant liquid by decanting and centrifuging. The filtrate contained only 0.02% of the original activity. The precipitate was extracted at 30°C. for one hour, first with 400 ml. of 10% NaCl solution, then twice with 200 ml. of 10% NaCl solution, and finally

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<sup>4</sup> The use of a cloth filter was unnecessary in these operations.

once with 200 ml. of 10% NaCl at pH 6.2 (adjusted with 0.5 N NaOH). A total volume of 1,150 ml. was obtained which contained 60% of the theoretical amount of enzyme present in the precipitate. Subsequent dialysis operations were carried out in a rocking dialysis machine, the solution containing enzyme being placed in a viscose sausage casing along with a marble to give constant stirring. Three additional cycles of the dialysis precipitation and extraction with decreased volumes of salt solution were carried out and the undissolved material left after each extraction removed by centrifuging. A 12 ml. solution containing 0.8856 g. of dry matter was obtained. The activity of this preparation was 146,500 PMU/g. of dry matter and 2,360 PMU/mg. of total nitrogen. The overall yield was, therefore, 40%. However, it should be pointed out that most of the loss occurred during the first dialysis and extraction cycle. Adjusting for the losses due to sampling at the different stages of operation, the *per cent* loss of activity in the later cycles was less than 10%. The PMU/mg. of total nitrogen had been raised from 278 in the combined filtrates to 2,360 in the final product. The PMU/g. of dry matter had been raised from 825 to 146,500. One-tenth ml. of the concentrate (146,000 PMU/ml.) was made up to 10 ml. with 10% NaCl solution in a 125 ml. Erlenmeyer, and distilled water added with stirring until the solution began to turn milky. A minute amount of additional NaCl solution was then stirred in to redissolve the forming precipitate and 95% ethanol slowly added until the mixture just became opalescent at 25°C. It was then warmed at 30°C. for one hour and set in a large beaker containing water at 30°C. and placed in a 0°C. cold room for several weeks. The precipitate which slowly settled out was collected by centrifuging and redissolved in 5 ml. of 10% NaCl. This solution contained a total of 135 PMU (27 PMU/ml.). The supernatant was found to contain 108 PMU/ml. in 100 ml. Total nitrogen and dry matter were run on this sample and it was found to contain 3,820 PMU/mg. N and 216,000 PMU/g. dry matter.

### *Procedure II*

The observation that, in fresh ground tomatoes, most of the PM is in the insoluble pulp, suggests another method by which a concentrated starting material for dialysis may be obtained. The pulp can be prepared from whole tomatoes (2) or it may be obtained as a waste product from tomato juice canning plants employing the cold break process. As no actual cannery waste was available at the time of these experiments a preparation simulating it was made from tomatoes which had been ground in the hammermill and frozen for storage. The frozen material was thawed and 500 ml. of it taken for the preparation. The serum was filtered from the suspended solids by suction and about 100 g. of wet solids obtained. The solids were washed once by stirring with 100 ml. of 0.05 N HCl solution and refiltered. Instead of using the extraction procedure wherein the pH is raised (2), the final residue was extracted overnight by standing with 100 ml. of 10% NaCl at 30°C. The extract was filtered off and the residue extracted twice more in the same way for 1 hour periods. The first two extracts obtained were combined and dialyzed free of Cl. The resulting precipitate was collected by centrifuging and redissolved by standing 4 hours with 15 ml. of the saline solution at 30°C. The material which remained undissolved by this treatment was centrifuged off and the clear extract again dialyzed until salt free. The precipitate was again collected and redissolved in 1 ml. of the saline solution. After the undissolved material was centrifuged off, the clear extract was found to

contain 3,060 PMU/ml., 2,300 PMU/mg. *N* and 207,000 PMU/g. dry material. The following table summarizes the steps in this procedure.

Small samples of the concentrates obtained were dried *in vacuo* over  $P_2O_5$  at room temperature and showed no significant loss in activity.

## DISCUSSION

Freezing of the tomato tissue improved the filterability and flocculation of the enzyme on dialysis. In experiments made to confirm the original observation of dialysis precipitation on material which had not been previously frozen, the amount of enzyme remaining in the filtrate varied from 10% to 50% as compared to less than 10% in the prefrozen samples.

It is difficult to explain the large initial loss of PM which occurs in the first dialysis precipitation-extraction operation, and why this loss does not occur in the later operations. Lack of its recurrence indicates that the loss is not due to inactivation by the dialysis or extraction but that possibly a portion of the enzyme becomes irreversibly precipitated or is occluded with inert particles.

The dry preparations obtained were some 100-fold more active than most active dry preparations previously described. MacDonnell, Jansen and Lineweaver (5) did report a solution of orange PM (purified by adsorption and elution from diatomaceous earth) which had an activity of 2,325 PMU and Jansen and MacDonnell (8) mention an orange pectin-esterase containing 2,790 PMU/mg. of protein nitrogen as compared with 3,800 PMU/mg. of total nitrogen in one of the dry preparations from this laboratory.

It is apparent that both of the methods described may give highly concentrated PM solutions. The saline extraction procedure seems to have the advantages of quicker reduction of the total volumes which have to be handled and requirement of fewer steps to reach comparable concentrations of the enzyme. However, in some laboratories it may be more convenient to employ freeze concentration rather than the saline extraction procedure.

Opinions vary concerning the presence in unblemished tomatoes of enzymes capable of hydrolyzing the polyuronide skeleton of pectin (4, 5). In any case, their presence would be of little significance in preparations intended for the demethylation of pectin because the activity of this enzyme or enzymes is negligible at the pH values ordinarily used in enzymatic demethylation.

## SUMMARY

Procedures have been described for preparing highly concentrated solutions of the enzyme pectin-methylesterase from tomatoes. These concentrates may be dried in vacuum over phosphorous pentoxide at room temperature without significant loss of activity.

TABLE I  
*Extraction of Pectin-Methylesterase from Tomato Pulp*

Sample	Total volume of extract ml.	PMU/ml.	Total PMU	Recovery of enzyme from pulp,* cumulative per cent; Recovery of enzyme from dialysis precipitates, per cent
First Extract	100	64.80	6480	70
Second Extract	100	12.60	1260	83
Third Extract	100	4.26	426	88
Dialysis Concentrates:				
First Concentrate	15	206	3085	40
Second Concentrate	1	3060	3060	99

\* The whole juice contained a total of 9,750 PMU and a total of 450 PMU were lost in the serum. The amount of enzyme which may have been lost in the acid wash was too small to detect. The *per cent* recovery of enzyme is, therefore, based on the amount calculated to be left in the solids after the serum had been removed.

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# Effect of Bulk, Casein and Fat in the Ration on the Utilization of Carotenes by White Rats

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## INTRODUCTION

Recent experiments by Fraps and Meinke (4) have shown that  $\alpha$ - or  $\beta$ -carotene dissolved in oil is digested by rats to the extent of about 60%, while in vegetables they averaged about 35%. Shaw and Deuel (7) reported that only 2% of the carotene dissolved in oil remained in the gut of white rats 42 hours after feeding. They suggest that the difference between the complete absorption reported in their tests and the incomplete absorption reported by other investigators may be due to the preliminary fasting period to which their rats were subjected. In other words, continuous intake of food may carry carotene from the intestines before digestion and absorption is complete.

This hypothesis raises the question whether rats fed on a bulky diet, digest, or utilize for storage of vitamin A in the liver, carotene to a lesser extent than those fed on a more concentrated diet. Too, the manner of feeding and the percentage of protein in the ration may also affect the digestibility and utilization of carotene.

## EXPERIMENTAL

Rats 28 days old from females fed a ration low in vitamin A were paired as to sex and litter in groups of 12 rats each in individual cages. One group received a ration containing corn meal or starch 59%, casein 22%, non-irradiated yeast 9%, cottonseed oil 4%, salt mixture 4%, irradiated yeast 1% and sodium chloride 1%. Other groups received a similar ration in which part of the corn meal or starch was replaced by the substance to be tested. Each group received purified crystalline carotene (1) dissolved in 0.1 cc. cottonseed oil. After the carotene had been fed for

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\* With the technical assistance of Jeanne De Mottier.

14 days, the rats were killed on the 15th day and the livers individually analyzed for spectrovitamin A (2, 3). The excrement was collected daily and placed in cold storage until analyzed. Crude carotene was determined in the excrement, and the carotenoid constituents of the crude carotene and the purified crystalline carotene ascertained by chromatographic adsorption on calcium hydroxide (6). The carotenes were expressed in terms of  $\beta$ -carotene equivalent (5). With the exception of one experiment, 60  $\gamma$  of  $\beta$ -carotene equivalent were fed daily. To test the effect of the constituents of the ration on the stability of carotene, 100 g. of several feeds were mixed with 2 g. of cottonseed oil containing 600  $\gamma$ /g. of purified carotene and the carotene determined. After incubation at 37°C. for 10 days, carotene was again determined. Starch and ground filter paper destroyed high percentages of carotene under these conditions. The rations and other nutrients used did not destroy the carotene. To avoid a possible deficiency, 0.5  $\alpha$ -tocopherol was added to 100 g. of the oils. The effect of bulky rations, as provided by cottonseed hulls and agar, the method of feeding and the effect of casein and fats of different kinds, was studied as shown in the table.

### RESULTS

The results are given in Table I. Although starch destroyed carotene when mixed with carotene dissolved in oil, the experiments in which starch was used gives results similar to those in which corn meal was used. Saliva and other digestive juices apparently inhibit the destructive action of starch. Replacing corn meal or starch by cottonseed hulls or agar produced a ration with lower productive energy, consequently smaller rats were obtained. The weights of the moist feces were very much greater when the cottonseed hulls or agar replaced corn meal or starch (Table I).

The apparent digestibility of the carotene was much less when cottonseed hulls replaced corn meal or starch in 5 of the 6 experiments, the difference being slight in one experiment; the livers were smaller but the parts per million of spectrovitamin A were larger. The quantity of spectrovitamin A stored in the livers, in most of the experiments, was apparently but little affected by the differences in apparent digestibility. The method of feeding the ration had little affect on the apparent digestibility of the carotene, but the storage of spectrovitamin A in the liver was less when the ration was withheld after the carotene was fed, indicating that the presence of digested food nutrients may have aided in storage of vitamin A in the livers.

The effect of the agar was different from that of the cottonseed hulls. The apparent digestibility of the carotene was slightly greater when 50% agar replaced corn meal, but was lower when cottonseed hulls were fed. The utilization of the carotene for storage in the liver was higher for the agar rations than for the corn meal ration.

Substitution of casein for part of the corn meal increased the apparent digestibility of carotene in all three of the experiments, although the effect of increasing the casein from 18% to 36% was slight and not in the same direction in one of the three experiments. Addition of the casein also increased the weights of the livers and the quantities of spectrovitamin A stored, the increase being in the same direction in all three of the experiments.

TABLE I

*Effect of Bulk, Protein Content and Method of Feeding upon Apparent Digestibility of Carotene and Storage of Vitamin A in Livers*

	Weight of feces	Apparent digestibility of carotene	Average weight of livers	Average spectro- vitamin A in livers	Spectro- vitamin A in liver
	g /period	per cent	(g.)	γ/g.	γ/liver
Ration removed at 8 A. M., carotene and then ration fed at 4:30 P.M.					
Corn meal ration	111	79.5	5.8	9.6	55.2
Cottonseed hulls 50%	1106	41.1	4.3	12.7	53.1
Corn meal ration	59	71.3	4.5	14.4	63.3
Cottonseed hulls 50%	651	69.6	3.5	17.7	61.9
Starch ration	63	74.8	3.8	11.3	60.8
Cottonseed hulls 50%	868	36.2	4.3	21.2	85.5
Ration removed at 8 A. M., carotene fed at 1 P. M., ration fed at 4:30 P. M.					
Starch ration	61	80.0	4.4	4.0	17.9
Cottonseed hulls 50%	1179	47.4	4.4	4.7	20.5
Ration removed at 8 A. M., 120 γ carotene fed at 8 A. M. next day, ration fed at 4:30 P. M.					
Corn meal ration	67	78.9	4.7	6.9	31.1
Cottonseed hulls 50%	434	53.9	2.7	7.9	20.8
Starch ration	33	87.2	3.4	6.9	23.6
Cottonseed hulls 50%	348	60.3	2.2	11.2	24.5



TABLE I—*Continued*

	Weight of feces	Apparent digestibility of carotene	Average weight of livers	Average spectro- vitamin A in livers	Spectro- vitamin A in liver
	g./period	per cent	(g.)	γ/g.	γ/liver
Ration removed at 8 A.M., carotene fed and then ration fed at 4:30 P.M.					
Corn meal	76	84.1	5.3	7.9	40.9
Agar 50%	1397	88.2	3.0	16.9	49.0
Corn meal	76	74.1	4.4	8.9	34.9
Agar 50%	700	78.0	3.1	15.9	48.0
Ration offered continuously					
Corn meal ration	96	62.8	3.5	9.7	33.8
Casein 18%	80	66.4	4.3	7.3	30.3
Casein 36%	74	67.7	4.3	9.7	41.2
Corn meal ration	82	71.8	4.0	8.6	33.4
Casein 18%	86	79.3	6.0	7.8	46.1
Casein 36%	73	77.6	6.2	10.7	63.6
Corn meal ration	59	64.2	2.9	15.8	43.8
Casein 18%	55	68.9	4.0	15.4	58.3
Casein 36%	47	70.9	4.4	15.2	64.1
Linseed oil 10%	69	69.6	4.1	10.4	42.2
Cottonseed oil 10%	64	73.4	4.3	10.8	45.2
Lard 10%	79	65.8	4.2	9.4	37.2
Linseed oil 10%	56	68.1	5.1	11.7	58.7
Cottonseed oil 10%	78	56.9	4.8	19.8	93.3
Lard 10%	69	59.8	4.7	17.5	82.2

The apparent digestibility of the carotene was erratic with the three oils. The storage of spectrovitamin A was highest with cottonseed oil, erratic with the other two. The livers were analyzed individually and considerable differences were found in the vitamin A content of livers fed on the same ration in the same experiment (5).

## ACKNOWLEDGMENT

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## SUMMARY

Carotene dissolved in oil fed with a bulky ration due to cottonseed hulls had a lower apparent digestibility than when fed with a similar ration containing corn meal or starch, but the storage of vitamin A in the livers was but little affected. With a bulky ration due to agar, the digestibility of the carotene was the same as with the corn meal ration. Delay in feeding the basal ration after feeding the carotene reduced storage of vitamin A in the liver. Substitution of casein for corn meal increased the apparent digestibility of carotene and storage of vitamin A in the liver. The apparent digestibility of the carotene was erratic with linseed oil, cottonseed oil and lard containing tocopherol.

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# Stereochemical Configuration and Provitamin A Activity.

## V. \* Neocryptoxanthin A

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### INTRODUCTION

The isomerization of the monohydroxy- $\beta$ -carotene, cryptoxanthin,  $C_{40}H_{55}OH$ , was observed by Zechmeister and Tuzson (9; cf. Strain 7). In those papers, as well as in subsequent studies of Fraps and Kemmerer (5), White, Zscheile and Brunson (8), Baumgarten, Bauernfeind and Boruff (1), *etc.*, only a single stereoisomer of cryptoxanthin was mentioned under the name of "neocryptoxanthin." It was shown, however, in collaboration with Lemmon (9) that under favorable conditions a minor isomer, neo-B, can be observed below the neo-A zone; furthermore, a neocryptoxanthin U, which is located above the all-*trans* compound in the calcium hydroxide column can also be observed. We believe that the "neocryptoxanthin" mentioned in current literature consisted mainly of neo-A but included a much smaller amount of neo-B.

The biological activity of "neocryptoxanthin" was first determined by Fraps and Kemmerer (5). Since they did not include some small zones located just below that of neocryptoxanthin, their results are directly comparable with ours. Fraps and Kemmerer found the ratio of the provitamin A potencies of  $\beta$ -carotene: cryptoxanthin: neocryptoxanthin as 1.1:0.6:0.4. The number of rats used and the levels administered were not reported.

\* For part IV see Reference 4.

† Contribution No. 1055.

## EXPERIMENTAL

The cryptoxanthin was obtained from persimmons, *Diospyros kaki* L., from which it had been isolated earlier by Schoen (6). We applied a different technique.

The fruits (which preferably should not be entirely ripe and soft) were ground and kept twice for several hours under methanol. After this dehydration, the liquid was eliminated in a basket centrifuge (diameter, 20 cm.), whereupon the paste, without previous drying, was extracted by two mechanical shakings with petroleum ether-methanol 3:1. For 18 kg. of fresh fruit, a total of 15 liters of the latter solvent were used. The extract was washed methanol-free, dried, concentrated to 1 liter, saponified with methanolic KOH over night, washed alkali-free, dried and developed on alumina-calcium hydroxide (1:4) (Alorco Grade F, - 80 mesh; Shell Brand lime, chemical hydrate, 98% through 325 mesh) columns with petroleum ether-acetone 9:1. The main zone, adsorbed in the upper section, was eluted with alcohol, rechromatographed and crystallized from petroleum ether-methanol. It was identified as cryptoxanthin by its spectrum, partition behavior, analysis and mixed chromatogram.

About 10 mg. of cryptoxanthin in 20 ml. of benzene was catalyzed with iodine and illuminated with two fluorescent Mazda lamps (3500°, 40 W; length of tube, 120 cm., distance from the solution, 60 cm.) for 3-5 minutes. The stereoisomeric mixture was then developed on a 17 × 1.9 cm. calcium hydroxide column (Shell Brand chemical hydrate, 98% through 325 mesh) with petroleum ether (b.p. 60-70°C.) containing 2.5% acetone. With the adsorbent now at our disposal neocryptoxanthin U which had been described earlier did not separate from all-*trans*-cryptoxanthin; however, its presence was revealed by the markedly shorter wave length maxima of the upper part of the main pigment zone. (A separation of neo-U was obtained by using benzene-petroleum ether mixtures as developer.) In every case, good separation of the all-*trans* form from neocryptoxanthin A took place. When the neo-A zone was cut out, its uppermost and lowest sections (the latter containing neo-B) were neglected. The main portion of neo-A was eluted with methanol and rechromatographed. Of course, the unchanged part of the all-*trans* form can be used in another isomerization experiment. About 4-5 mg. of chromatographically homogeneous neocryptoxanthin A was used for each concentrate.

The benzene solution of neocryptoxanthin A showed in the Beckman photoelectric spectrophotometer a main maximum at 458  $m\mu$ . The corresponding figures are, for the all-*trans* form 464  $m\mu$ , and for the iodine-catalyzed stereoisomeric mixture, 461  $m\mu$ . In the Wesson oil used in the bioassays the maximum extinction of neocryptoxanthin A was located at 455  $m\mu$ . When such a solution was kept at 4°C., in darkness, the position and height of this maximum remained constant for several days and then, within 10 days, gradually migrated about 1  $m\mu$  towards longer wave lengths. The sharpness of the curve was not diminished. Since the main maximum of all-*trans*-cryptoxanthin in the oil is located at 463  $m\mu$ , only about 1/10 of the *cis* compound was sterically altered during the storage period. Consequently, concentrates for the bioassays were renewed about every eighth to tenth day.

The concentration of neocryptoxanthin A in Wesson oil was obtained as recently described for neo- $\beta$ -carotene B by using the extinction values of the iodine-catalyzed

mixture (3). The following figures are valid for neocryptoxanthin A in the oil:  $E_{1\text{ cm}}^{1\%} = 1560$ ; and the concentration ( $\gamma/0.1\text{ ml.}$ ) =  $0.64 \times E_{1\text{ cm.}}$ .

The bioassays were carried out as described earlier (3, 4). The rats used were from our stock colony. Supplements were administered orally by means of a hypodermic syringe with a blunt needle. The supplements were made up in cottonseed oil in such concentration that the dose was present in 0.1 ml. The daily intake of  $\alpha$ -tocopherol of 0.5 mg. was included in the administered oil. The supplement was given six times weekly (Sunday being omitted) except when rats were started on the assay on a Sunday. In this case the supplement was omitted on a later day of the first week.

## RESULTS

Table I gives a summary of the data on the 122 rats used in this test. All-*trans*- $\beta$ -carotene was given at daily levels of 0.4 and 0.8  $\gamma$  while neocryptoxanthin A was administered at 0.8, 1.6 and 3.2  $\gamma$ .

The average growth response for 0.8  $\gamma$  of  $\beta$ -carotene was 37.6 g., which gives a corrected value of 41.6 g. when adjusted for the fact that it was administered only 24 days instead of 28 days. This figure is practically identical with the average of the previous series of tests on carotenoids where  $\beta$ -carotene has been used as a standard (3,4). The mean value calculated from the log dose-body weight curve for the 0.8  $\gamma$  dose was 42.2 g. The value for the slope of the curve which gives an index of the relative response at various levels of carotene was 0.0593 compared with an average value for the six tests of 0.0643.<sup>1</sup>

When neocryptoxanthin A was fed at a level of 0.8  $\gamma$ , 8 of the 19 animals died before the 28th day while the average gain of the survivors was 16.0 g. When the level of neocryptoxanthin was increased to 1.6  $\gamma$ , only 3 animals died and the average net gain was 31.2 g. At the highest dose (3.2  $\gamma$ ) one rat died but the gain was 41.8 g. In the case of the negative controls, which included one rat from each of the 29 litters, 17 died before the 28th day and all except one of the remaining twelve rats were losing weight. The survivors lost, on an average, about 4 g.

The dosage/gain-in-weight curves are given in Fig. 1.

The curve connecting the two lowest dosages of neocryptoxanthin A and that connecting the points for  $\beta$ -carotene are reasonably parallel.

<sup>1</sup> Calculated from the expression

$$b \text{ (slope)} = \frac{y - y^1}{\log x - \log x^1}$$

Where  $y$  and  $y^1$  are the gain in weight at the different levels of carotene intake and  $\log x$  and  $\log x^1$  are the logs of the respective doses of carotene.

TABLE I  
*Summary Table of Bioassay Experiments on Male and Female Rats Receiving All-trans- $\beta$ -carotene, Neocryptoxanthin A in Cottonseed Oil or the Oil Alone (Negative Controls)*

(The average results on males and females are weighted equally. Where animals died in the course of the experiment, the number of animals still alive, which is included in the average, is given in parentheses. The average age at the start of the depletion period was 22-23 days.)

Supplement	Dose per day	Number of rats		Depletion period			Assay period						
							Average increase in body weight up to following days						Average final weight
		Male	Female	Average weight at start	Average duration	Average final weight	5th	10th	15th	20th	25th	28th	
All- <i>trans</i> - $\beta$ -carotene	0.4	8	10	<i>g.</i> 41.8	<i>days</i> 25.9	<i>g.</i> 98.2	<i>g.</i> 3.4	<i>g.</i> 9.2	<i>g.</i> 11.8	<i>g.</i> 19.2 (16)	<i>g.</i> 22.4 (15)	<i>g.</i> 19.8 (15)	<i>g.</i> 117.8
	0.8	8	12	42.2	23.9	93.8	6.0	16.0	25.4	30.6	36.0 (19)	37.6 (19)	130.8
Neocryptoxanthin A	0.8	8	11	42.0	24.4	96.4	4.6	8.8 (18)	15.5 (16)	17.3 (14)	14.4 (14)	16.0 (11)	111.6
	1.6	8	11	42.6	24.6	96.2	3.4	9.0 (18)	15.6 (18)	21.2 (18)	23.1 (18)	31.2 (16)	126.1
	3.2	7	10	42.0	24.5	95.7	8.7	16.2	27.8 (16)	34.2 (16)	37.8 (16)	41.8 (16)	138.2
Negative controls	0.0	14	15	42.9	24.6	98.2	0.6 (28)	0.2 (24)	1.5 (20)	2.0 (16)	3.0 (12)	-4.2 (12)	87.5

In the two lower dosages full use of the neocryptoxanthin very probably occurred since the dosage/gain in weight curve is practically parallel to that for  $\beta$ -carotene. When neocryptoxanthin was given at the highest level, the point falls somewhat below the line for the lower points. This would indicate that at this level neocryptoxanthin A was not fully used.

The potency of neocryptoxanthin as calculated by the procedure of Coward (2) was found to be 39 and 44% in the 1.6 and 0.8  $\gamma$  tests, respectively, of that of all-*trans*- $\beta$ -carotene or 71% of that of all-*trans*-

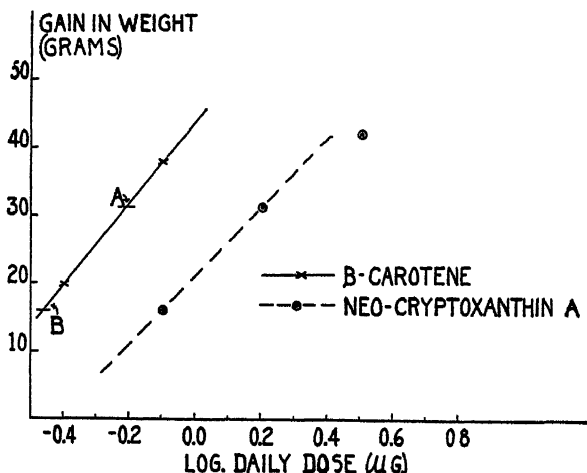


FIG. 1

Relationship of Gain in Weight to log of Daily Dosage of  $\beta$ -Carotene and Neocryptoxanthin A

Points A and B represent the projection on the  $\beta$ -carotene curve of the average gain (in grams) for the groups receiving 1.6  $\gamma$  and 0.8  $\gamma$  of neocryptoxanthin A respectively.

cryptoxanthin, since the potency of the latter amounts to 58% of that of all-*trans*- $\beta$ -carotene (4). This result is in excellent agreement with that reported by Fraps and Kemmerer (5), from whose data the respective percentages 67% and 55% follow.

Neocryptoxanthin A has been tentatively assigned a steric configuration which postulates the presence of two *cis* double bonds, one being located at the center of the chromophore (9). It is remarkable that the rearrangement, all-*trans*-cryptoxanthin  $\rightarrow$  neo A causes only the moderate loss of about 30% in the provitamin A potency. In



contrast, in the course of the conversion, all-*trans*- $\beta$ -carotene  $\rightarrow$  neo- $\beta$ -carotene B, about 50% of the bioactivity is lost, although the two *cis* compounds mentioned seem to possess similar configurations (3). Indeed, if we disregard the specially built poly-*cis* compound, pro- $\gamma$ -carotene, neocryptoxanthin A appears at the present time to be that *cis* carotenoid which in the strength of its provitamin A activity stands nearest to the corresponding all-*trans* form.

### SUMMARY

The provitamin A activity in rats of neocryptoxanthin A was found to amount to 71% of that of all-*trans*-cryptoxanthin or 42% of that of all-*trans*- $\beta$ -carotene.

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# Enzymatic Mechanisms in the Respiration of Spinach Leaves

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## INTRODUCTION

Our present knowledge of the respiration of plants is based primarily on studies of overall gas exchange. To a lesser extent studies have also been made of individual enzymes and of individual enzymatic reactions of apparent importance in respiration. For no plant tissue, however, has it been possible to present an integrated picture of the course of the whole respiratory process from hexose to carbon dioxide and water. In this paper the respiration of the spinach leaf will be considered from such an overall standpoint. Such treatment is possible, of course, only because the advanced state of knowledge concerning the respiratory mechanisms of microorganisms and of animal tissues allows us to verify the presence or absence of similar or identical mechanisms in the higher plant. The present paper is not intended to be a detailed study of each enzyme system, but rather to indicate the general types of enzyme systems concerned in the respiration of spinach leaves. It is hoped that this survey may be followed by a more intensive investigation of the individual systems involved.

## METHODS

### *Plants*

Spinach leaves were obtained fresh from field grown plants, from plants grown for the purpose under laboratory conditions and from plants obtained from the local markets. No systematic differences in respiratory behavior were noted between leaves from these three sources, with the exception that the market leaves behaved as though partially depleted of reserve carbohydrate, as would be expected from leaves which had been stored for a short time. Such market leaves were, in general, the source of the material for the experiments detailed below. Mature leaves were selected throughout.

*Gas Exchange Measurements*

Gas exchange measurements were made in the Warburg respirometer, using conical vessels of approximately 16 cc. volume. Oxygen uptake was measured in vessels having concentrated KOH in the central well, while the difference between O<sub>2</sub> uptake and CO<sub>2</sub> evolution was measured in a second vessel without KOH in the usual way. All of the measurements were made at 30°C. For the gas exchange measurements, 4-5 leaves were cut with sharp scissors into pieces 5-10 mm.<sup>2</sup> in area. These fragments were thoroughly mixed and 200 mg. samples were then weighed out into each vessel. Two cc. of buffer, or of buffer containing *addendum*, was then added. Duplicate samples of leaf were found to give highly reproducible rates of gas exchange, the rates agreeing, in general, to within 5%.

Penetration of added substrates or inhibitors into the leaf tissue was achieved by vacuum infiltration. The vessels containing the leaf fragments and test solutions were placed in a vacuum desiccator and the whole evacuated twice, air being slowly readmitted each time. Preliminary experiments showed that such infiltration exerted no effect on the respiratory rate, *i.e.*, that this procedure was not injurious to the tissue.

## EXPERIMENTAL

*Preliminary Experiments*

Fresh spinach leaves prepared as described above and suspended in 0.1 *M* phosphate buffer of pH 4.5 are able to respire for at least three hours without decrease in rate, with a respiratory quotient of approximately unity, as is shown in the experiment of Table I. The leaves

TABLE I  
*O<sub>2</sub> Uptake and CO<sub>2</sub> Evolution by Spinach Leaf Sections  
Suspended in Phosphate Buffer*

Expt. No.	Gas	Gas exchange in mm. <sup>3</sup> /200 mg. leaf/hr.		
		First hr.	Second hr.	Third hr.
E-02; leaves fresh from field	O <sub>2</sub>	123	125	130
	CO <sub>2</sub>	123	126	131
	R.Q.	1.0	1.0	1.0
E-103; leaves depleted 4 days at 0°C.	O <sub>2</sub>	82	75	62
	CO <sub>2</sub>	61	58	51
	R.Q.	.74	.77	.82

as picked from field plants during the day apparently contain sufficient carbohydrate substrate to permit them to respire for an extended period. In the case of partially depleted leaves, a dropping off of

respiratory rate with time is found and, in addition, the respiratory quotient is significantly less than unity. It is worth noting that, despite the high acid content of spinach leaves (in the neighborhood of 5% of oxalic acid on a dry weight basis), the tissue does not show the abnormally low respiratory quotient characteristic of the leaves of succulents.

Table II shows the influence of the pH of the buffer in which spinach leaves are suspended on the rate of O<sub>2</sub> uptake and on their CO<sub>2</sub> evolution. Buffers with a pH higher than 5.0 bind appreciable amounts of CO<sub>2</sub> and this bound gas was released at the end of the experimental period by addition of a slight excess of acid from the side arm of the

TABLE II  
*Influence of pH on Respiratory Rate and Respiratory Quotient of Spinach Leaves*  
(E-110. Rate for 1st hour)

pH	Gas exchange in mm <sup>3</sup> /200 mg leaf/hr			Total CO <sub>2</sub>	R Q
	O <sub>2</sub> taken up	CO <sub>2</sub> evolved	CO <sub>2</sub> bound		
4.5	86	80	0	80	0.93
5.0	84	77	2	79	0.94
5.5	82	72	4	76	0.93
6.0	81	61	5.5	66.5	0.82
6.5	82	52.5	10.5	63	0.77
7.0	81	35	24.5	59.5	0.73

vessel. It may be seen that, although pH has only a small influence on the rate of O<sub>2</sub> uptake over the limits studied, there is a significant depression of the respiratory quotient at the higher pH values. This phenomenon has also been observed during the study of the respiration of *Avena* coleoptiles (7) and no explanation has as yet been found for it. In all experiments with living leaves, the pH was kept at 4.5 unless otherwise noted.

### *The Terminal Oxidase*

The enzyme responsible for the actual uptake of O<sub>2</sub> in plant respiration has been the subject of much investigation since Bach and Chodat (13) sought to identify this factor with the enzyme which is now known as polyphenol oxidase. Brown and Goddard (10) and Goddard (15) have established with certainty that the terminal oxidase of the wheat

embryo is cytochrome oxidase as it is in animal tissues, and Goddard has separated from wheat embryo cytochrome *c*, which is apparently identical with that of beef heart. That cytochrome oxidase is also involved in the respiration of other plant tissues has been indicated by Okunuki (31, 32, 33, 34) for pollen and by Marsh and Goddard (30) for carrot root. James and Cragg (23) have suggested, on the basis of incomplete experiments, that ascorbic acid oxidase may be the terminal oxidase of barley leaves and that ascorbic acid may function as a carrier between the oxidase and reduced coenzyme I. The work of Boswell and Whiting (9), Boswell (8) and of Baker and Nelson (2) leaves no doubt, however, that, in the potato tuber, the bulk of the  $O_2$  uptake in respiration does indeed pass through polyphenol oxidase. That the same is true of spinach leaves is shown by the experiments described below.

Spinach leaves contain a highly active polyphenol oxidase, as can be demonstrated *in vitro*, either with preparations of whole leaves ground to a *brei* in a blender, or preferably, with whole cytoplasm of spinach leaves prepared after the method of Wildman and Bonner (39). Whole cytoplasm as shown in Table III can carry out rapid oxidation of

TABLE III

*Polyphenol Oxidase Activity of Whole Spinach Cytoplasm*

(50 mg. Whole cytoplasm per vessel (approximately 10 mg. protein). pH 6.5.

Experiment E-122. Total volume of solution: 2 cc. per vessel)

Vessel No.	Mg. catechol	Inhibitor	Conc. of inhibitor	Oxygen uptake mm <sup>3</sup> /vessel/hr. during first 10 min.
1	3	None	0.0000 <i>M</i>	530
2	3	<i>p</i> -Nitrophenol	0.01 <i>M</i>	118
3	3	<i>p</i> -Nitrophenol	0.001 <i>M</i>	288
4	3	<i>p</i> -Nitrophenol	0.0001 <i>M</i>	433
5	3	<i>o</i> -Nitrophenol	0.01 <i>M</i>	242
6	3	<i>o</i> -Nitrophenol	0.001 <i>M</i>	450

catechol and can also oxidize *p*-cresol, although at a slower rate. Oxidation of either substrate is inhibited by *p*-nitrophenol, a compound which may be considered as an analogue of the normal substrate of the enzyme. The related *o*-nitrophenol, an analogue of *o*-phenols which are not attacked by polyphenol oxidase, is approximately 10 times less inhibitory to the action of the enzyme than the *p*-isomer. Although

polyphenol oxidase is present in the spinach leaf, no evidence could be found that either ascorbic acid oxidase or cytochrome oxidase exist in the tissue. Neither could any evidence be found for the existence of cytochrome among the purified leaf proteins.

The respiration of spinach leaves is inhibited by HCN at relatively high concentrations (Table IV). Even in the presence of 0.01 *M*

TABLE IV  
*Influence of Cyanide on the Respiration of Spinach Leaf Section*  
(pH 4.5. Experiment E-76)

Treatment	O <sub>2</sub> consumption $\overline{\text{mm.}}^3/200 \text{ mg. leaf/hr.}$				R.Q.	Per cent inhibition
	First hr.	Second hr.	Thurd hr.	Fourth hr.		
Control	143	138	129	111	0.9	—
HCN 0.01 <i>M</i>	99	60	43	28	1.0	75
HCN 0.001 <i>M</i>	94	74	63	54	1.0	49

cyanide, however, the leaves remained turgid and appeared healthy after 4 hours. The need for high concentrations of HCN may be related to a slow penetration of the material into the cells even after infiltration as is indicated by the fact that inhibition continued to increase with time even after 4 hours. The sensitivity of both O<sub>2</sub> uptake and CO<sub>2</sub> evolution to cyanide may be taken to indicate that a heavy metal catalyst is involved. Both polyphenol oxidase and cytochrome oxidase are known to be inhibited by cyanide. The study of CO inhibition and the reversal or non-reversal of such inhibition in light would, in general, permit of a diagnosis as to which catalyst is involved. In the present case this method cannot be applied because of the complications inherent in a photosynthetically active tissue.

If spinach leaves are infiltrated with catechol, CO<sub>2</sub> production is greatly decreased, in fact may cease altogether (Table V). Oxygen consumption is initially increased, but rapidly decreases until it also falls below the level of the control, untreated leaves. It has been shown by Boswell and Whiting (9) and confirmed by others, that the action of polyphenol oxidase on catechol results not only in oxidation of the substrate but also in rapid inactivation of the enzyme. This fact accounts for the transitory nature of the rise in oxygen consumption when catechol is supplied. The decrease in rate of CO<sub>2</sub> production after

was increased by 119% over the same period. The fact that the addition of dihydroxyphenylalanine causes increases in the  $\text{CO}_2$  evolution strongly indicates a linkage between polyphenol oxidase and the  $\text{CO}_2$ -producing mechanisms of the leaf.

#### *The Initial Breakdown of Hexose*

The early work on the mechanism of yeast fermentation stimulated and found a counterpart in work on fermentation carried out with the tissues of higher plants. Iwanoff (21), Kostytshev (27) and others have shown that some tissues, as pea seed meal, possess systems for the fermentation of hexose to alcohol and  $\text{CO}_2$ . That the rate of pea seed meal fermentation is influenced by phosphate concentration has been shown by Bodnár and Hoffner (6). More recent work has shown that higher plants contain the same hexose phosphates which occur as intermediates of glycolysis in yeast or muscle. Thus, fructose diphosphate has been found in pea seed meal (37) and oat coleoptiles (1). Fructose-6-phosphate has been shown to occur in pea leaves (20) and in sugar beet leaves (11). Glucose-6-phosphate has been found in pea leaves (20) and in sugar beet leaves (11). With the exception of phosphorylase (19, 17), the enzymes of the initial carbohydrate breakdown have, however, been but little studied in higher plants.

Perhaps the most extensive study of carbohydrate breakdown in leaves is that of James and co-workers (22, 23, 24, 25, 26). This group has established that hexose diphosphate, phosphoglycerate and pyruvic acid are intermediates in the normal breakdown of hexose by leaves of barley.

The following experiment indicates that spinach leaves contain enzymatic systems for the conversion of hexose to fructose diphosphate and to phosphoglyceric acid. Fresh spinach *brei* was prepared by blending 500 g. of spinach leaf in 100 cc. of water and filtering through muslin. To the *brei* was added either glucose (to make 1%), glucose and adenosine triphosphate (ATP) (to make a concentration of 1 mg./cc.), or nothing (control). The three preparations were then incubated at 24°C. Aliquots were removed after various periods of incubation and fructose diphosphate and phosphoglyceric acid determined by the methods of Umbreit *et al.* (38).

The results, which are recorded in Figs. 1 and 2, show an initial formation of fructose diphosphate from glucose, particularly in the presence of ATP. The fructose diphosphate thus formed slowly disappears again with time. As shown also in Fig. 1 the leaf *brei* initially contains an appreciable amount of fructose diphosphate. Phosphoglycerate was similarly formed in the presence of leaf *brei* and dextrose, and the amount accumulated was also increased somewhat in the presence of ATP. The formation of these two key intermediate substances by spinach leaf *brei* from hexose, together with the apparent

influence of ATP on the system suggests that a glycolytic system similar to that found in yeast and muscle may also exist in the spinach leaf. This conclusion is further supported by the following *in vivo* experiments.

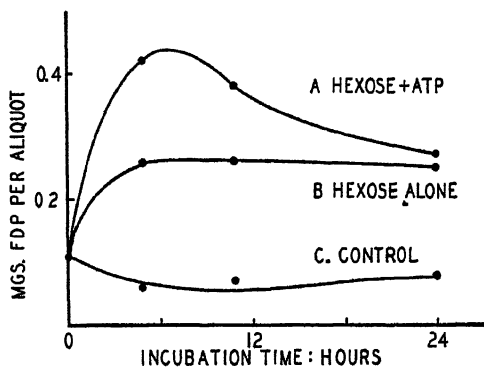


FIG. 1

Production of Fructose Diphosphate from Hexose in Spinach Leaf *brei*  
Incubated at 24°C.

The reaction mixture A contained per 10 cc.: *brei* representing 50 g. leaf, 0.01 *m* phosphate buffer, 100 mg. dextrose, 10 mg. ATP. B contained no ATP, while C contained neither ATP nor hexose. Ordinates represent amount of fructose diphosphate/10 cc. aliquot.

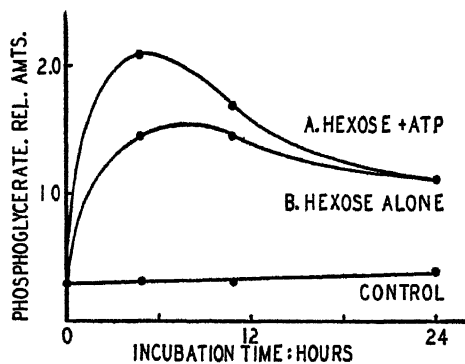


FIG. 2

Production of Phosphoglyceric Acid from Hexose in Spinach Leaf *brei*  
Incubated at 24°C.

Reaction mixtures identical with those of Fig. 1



The respiration of intact spinach leaves is inhibited by fluoride, the inhibition affecting  $\text{CO}_2$  evolution as well as  $\text{O}_2$  uptake (Table VIII). Fluoride inhibition, although not completely specific, can frequently be attributed to inhibition of the enzyme enolase which is responsible

TABLE VIII  
*Inhibition of Respiration of Spinach Leaf Sections by Sodium Fluoride*  
(Experiment E-95)

Treatment	Gas exchange $\bar{\text{m}}\bar{\text{m}}.^3/200$ mg. leaf/hr.			Per cent inhibition
	hr. 1	hr. 2	hr. 3	
$\text{O}_2$ Control	147	141	140	—
+1 mg. NaF/cc.	51	22	16	89
$\text{CO}_2$ Control	133	139	141	—
+1 mg. NaF/cc.	67	28	19	87

for the conversion of 2-phosphoglycerate to pyruvic acid. Thus, fluoride may inhibit the formation of pyruvic acid from hexose. Fluoride inhibits respiration at approximately this level in spinach leaves as is shown by the experiments of Table IX. The respiration of leaves poisoned with fluoride can be quantitatively restored by the further

TABLE IX  
*Inhibition of Respiration of Spinach Leaves by Sodium Fluoride and Reversal of the Inhibition by Pyruvate*

Expt.	Treatment	$\text{O}_2$ consumption in $\bar{\text{m}}\bar{\text{m}}.^3 \text{ O}_2/200$ mg. leaf/hour		
		hr. 1	hr. 2	hr. 3
E-137	Control	91	90	90
	+NaF, 0.1 mg./cc.	45	23	19
	+NaF+pyruvate 2.5 mg./cc.	102	88	85
E-149	Control	78	72	76
	+NaF, 0.1 mg./cc.	75	36	28
	+NaF+10 mg. glucose/cc.	64	34	29
	+NaF+5 mg. pyruvate/cc.	88	71	75

addition of pyruvic acid, that is, fluoride-poisoned leaves can utilize pyruvic acid as well as the unpoisoned leaf can utilize the normal endogenous substrates. Fluoride-poisoned leaves cannot, however, utilize glucose (Table IX). It seems, therefore, that in spinach leaves, as in yeast and in animal tissues, it is a process leading to the production of pyruvic acid which is poisoned by fluoride. This fact, taken together with those presented in Figs. 1 and 2, indicates that hexose breakdown in the spinach leaf may occur through phosphorylated intermediates with the ultimate production of pyruvic acid. Actual isolation of pyruvic acid from respiring leaves has been accomplished by James and James (25) with barley and by Bennett with onion (4).

### *Dehydrogenases of Spinach Leaves*

The respiration of spinach leaves is inhibited by monoiodoacetic acid, as is shown in Table X, CO<sub>2</sub> production, in particular, being

TABLE X  
*Effect of Iodoacetate on the Respiration of Spinach Leaves*  
(Experiment E-82. Third hour after infiltration)

Treatment	Gas exchange in mm <sup>3</sup> /200 mg. leaf/hr			
	O <sub>2</sub>	CO <sub>2</sub>	Per cent inhibition O <sub>2</sub>	Per cent inhibition CO <sub>2</sub>
Control	105	105	—	—
+1 mg. iodoacetate/cc.	11	3	90	97
+0.1 mg. iodoacetate/cc.	40	16	62	85

strongly influenced. Iodoacetate is known to inhibit numerous dehydrogenases, particularly phosphoglyceraldehyde dehydrogenase, malic dehydrogenase and alcohol dehydrogenase. Commoner and Thimann (14) have indicated that iodoacetate inhibits the respiration of *Avena* coleoptiles through an influence on organic acid metabolism. Iodoacetate is, however, sufficiently non-specific in its action that no great stress can be laid on its activity in the present case in relation to any particular enzyme. That spinach leaves do contain dehydrogenases can be shown directly.

Whole cytoplasmic protein of spinach leaf was prepared according to the methods of Wildman and Bonner (39). Portions of this protein were tested for dehydrogenase

activity using the Thunberg technique. All of the dehydrogenase experiments were carried out at a pH of 6.7, in 0.025 *M* phosphate buffer. To 1 cc. of the enzyme preparation was added 1 cc. of a solution of coenzyme preparation made from yeast and containing both coenzymes I and II (but no appreciable amount of substrate), 0.1 cc. of a solution containing 1 mg. of the desired substrate, and 0.1 cc. of a solution containing 0.25 mg. of thionine. The tubes were evacuated and incubated at room temperature. The time needed for 90% reduction of the dye was determined by comparison with a similar non-evacuated solution containing a ten times lower concentration of the dye.

Table XI shows that the cytoplasmic protein is able to reduce thionine at the expense of isocitric acid, and that the oxidation of isocitric acid proceeds only in the presence of a coenzyme. Since the

TABLE XI

*Presence of Isocitric Dehydrogenase in Whole Spinach Cytoplasm*  
(E-77. pH 6.7. Temperature 24°C. Thunberg technique)

Amt. of cytoplasm	Coenzyme preparation	Isocitrate	Total vol.	Time needed for 90 per cent reduction
mg.	cc.	mg.		min.
50	1	1.0	2.2	12
50	None	1.0	2.2	More than 180
50	None	None	2.2	More than 180
None	1	1.0	2.2	More than 180

preparation of coenzyme contained both coenzymes I and II, it is not possible to state which coenzyme is required by the enzyme of spinach. Table XII shows that spinach leaf cytoplasmic protein contains other

TABLE XII

*Reduction Times of Various Substrates in the Presence of Whole Spinach Cytoplasmic Protein*

(Thunberg technique. Temp. 24°C. pH 6.7. Total volume 2.2 cc./tube)

Protein	Coenzyme	Substrate	Substrate	Time needed for 90 per cent reduction
mg.	cc.		mg.	min.
10	1	Isocitrate	1 mg.	14
10	1	Citrate	1 mg.	30
10	1	Malate	1 mg.	40
10	1	Glutamate	1 mg.	30
10	1	Ethyl alc.	1 mg.	70
10	1	None	—	over 180
0	1	None	—	over 180

dehydrogenases in addition to that for isocitrate. Enzymes for the oxidation of malate, glutamate and ethyl alcohol also appear to be present. In addition, the enzyme aconitase which converts citric acid to isocitric must also be present, since citric acid is oxidized, although at a rate somewhat slower than found for isocitric.

### *Succinic Dehydrogenase and the Krebs Cycle in Spinach Leaves*

The information adduced above indicates that pyruvic acid may be an intermediate metabolite in the respiration of hexose by spinach leaves. A mechanism for the oxidation of pyruvate has been proposed by Krebs and Johnson (29) and summarized by Krebs (28).

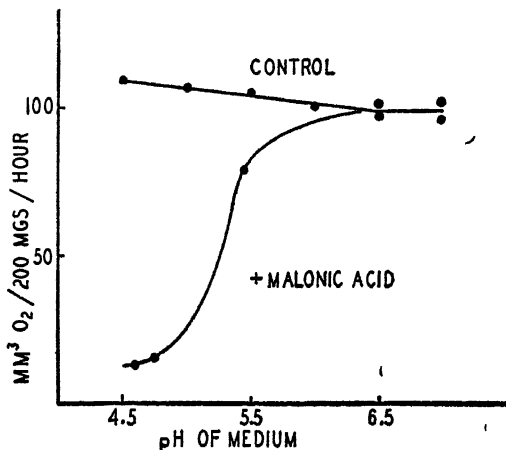


FIG. 3

Effect of pH on the Inhibition of Spinach Leaf Respiration by Malonate.  
Inhibitor concentration 5 mg./cc. throughout

In this mechanism, pyruvate is combined with oxaloacetate to yield, ultimately, isocitrate and  $\text{CO}_2$ . The isocitrate thus produced is oxidized and decarboxylated through  $\alpha$ -ketoglutarate and succinate to oxaloacetate. This chain of reactions, in which pyruvate is oxidized to  $\text{CO}_2$  and water through the intermediary of the plant acids, is known as the Krebs cycle and the evidence that a mechanism of this type is a principal path of carbohydrate oxidation in animal tissues is not inconsiderable. All of the acids which participate in the Krebs cycle are known from higher plants. Certain of the enzymes have also been sought and found in higher plant tissues, notably aconitase, isocitric and malic dehydrogenases and fumarase. Although the participation of a mechanism similar to the Krebs cycle in the respiration of higher plants has frequently been suggested, for example by Chibnall (12), as yet no good

evidence for the existence of such a system in a higher plant tissue has been brought forward. The data to be presented below show that organic acids participate in the respiration of spinach leaves and indicate that cyclic reactions generally similar to those of the Krebs system may be operative.

Under certain conditions spinach leaves are able to utilize organic acids in respiration. It has been mentioned above that leaves fresh from the field-grown plant do not appear to be limited in their respiration by substrate concentration. Leaves were, therefore, starved at room temperature in the dark for 24–48 hours, or for 4 or more days at 0–2°C. These leaves, which have presumably been partially depleted of carbohydrate reserve, respire at a lowered rate, their rate of respiration drops off rapidly with time, and they typically exhibit a lower respiratory quotient than is found in the case of fresh leaves. When such leaves are supplied with glucose, the rate of respiration is increased, CO<sub>2</sub> production in particular being affected so that the respiratory quotient is raised to approximately unity (Table XIII).

TABLE XIII

*Effects of Glucose and of Various Organic Acids on the Rate of Respiration of Partially Depleted Spinach Leaves*

Added compound	Concentration	Expt. No.	Length of starvation period	Increase in gas exchange (per cent of control)		Respiratory quotient	
				O <sub>2</sub>	CO <sub>2</sub>	Control + substrate	
Glucose	10 mg./cc.	104	4 days at 0°	28	59	.80	.99
Glucose	10 mg./cc.	105	20 hrs at 24°	34	64	.77	.94
Succinate	1 mg./cc.	103	4 days at 0°	21	57	.82	1.06
Succinate	1 mg./cc.	104	4 days at 0	29	60	.80	1.13
Succinate	1 mg./cc.	105	20 hrs at 24°	43	79	.77	.97
Malate	1 mg./cc.	106	22 hrs at 24°	18	98	.51	.85
Fumarate	1 mg./cc.	107	24 hrs at 24°	20	26	.83	.88
Isocitrate	1 mg./cc.	106	22 hrs at 24°	11	63	.51	.75
Citrate	1 mg./cc.	107	24 hrs at 24°	34	48	.83	.92
Pyruvate	1 mg./cc.	109	26 hrs at 24°	13	32	.83	.97

Depleted leaves also respond with increased O<sub>2</sub> uptake and CO<sub>2</sub> output to the addition of succinic, malic, fumaric, isocitric, citric and pyruvic acids, as may be seen in Table XIII. These acids can support increased respiration over a period of several hours. Similar effects of organic acids have been found with potato tuber by Bennet-Clark and Bexon

(3). Addition of organic acid did not, however, augment the rate of oxidation of hexose as has been reported by Gözsy and Szent-Györgyi (16) and others for pigeon breast muscle.

The respiration of spinach leaves is strongly inhibited by malonic acid. The effect of added malonate depends not only on the concentration of the inhibitor, but also on the pH of the medium in which it is infiltrated, as is shown in Fig. 3. Malonate in the concentration of 5 mg. of the acid/cc. infiltrated at a pH of 4.5 in phosphate buffer inhibited  $O_2$  uptake by 80%. The same concentration infiltrated in buffer at pH 7.0 was without inhibitory effect. Solutions of intermediate pH's possessed intermediate inhibitory effects. The shape of the pH *vs.* inhibition curve for malonate suggests a titration curve having an inflection point in the neighborhood of pH 5 to 5.5. The dissociation constant for the second hydrogen of malonic acid is 5.3. These facts may be interpreted on the assumption that the influence of pH in the present case is related to the penetration of malonic acid into the individual cells of the tissue, penetration being proportional to the concentration of the monobasic malonate ions.

Malonate has been shown by Quastel and Wooldridge (35) and others to be a specific inhibitor of the enzyme succinic dehydrogenase. The fact that malonate inhibits the respiration of spinach leaves may be taken, according to Krebs (28), as indicating the participation of this enzyme in respiration. It should be noted (Table XIV) that malonate inhibits not only  $O_2$  consumption of spinach leaves by 90% or more,

TABLE XIV  
*Effect of Malonic Acid on the Respiration of Spinach Leaves*  
(Experiment 91-92)

Treatment		Rate of gas exchange $\text{mm.}^3/200 \text{ mg. leaf/hr.}$			Per cent inhibition	R.Q.
		hr. 1	hr. 2	hr. 3		
$O_2$	Control	133	121	129		
	+5 mg. malonate/cc.	39	16	9	93	
	+1 mg. malonate/cc.	87	71	68	47	
$CO_2$	Control	129	124	129		1.0
	+5 mg. malonate/cc.	47	19	12	91	1.3
	+1 mg. malonate/cc.	94	80	77	40	1.1

but that it also reduces  $\text{CO}_2$  evolution by approximately the same amount. Succinic dehydrogenase is, therefore, a central enzyme not only from the  $\text{O}_2$  uptake standpoint, but is also an essential part of the  $\text{CO}_2$ -liberating mechanism.

The inhibiting effect of malonate on spinach leaf respiration can be partially overcome by the addition of succinic acid as is shown in Table XV. Additions of as little as 1 mg. of succinic acid per cc. resulted in

TABLE XV

*Influence of Malonic Acid on the Respiration of Spinach Leaves and the Influence of Other Acids on This Inhibition*

Expt.	Treatment	$\text{O}_2$ uptake in mm. <sup>3</sup> /200 mg. leaf/hr. third hour of treatment
E-138	Control	73
	+ 5 mg. malonate/cc.	18
	+ 5 mg. malonate/cc.+1 mg. succinate/cc.	58
	+ 5 mg. malonate/cc.+1 mg. fumarate/cc.	78
E-278	+20 mg. malonate/cc.+5 mg. fumarate/cc.	16
E-135	+ 5 mg. malonate/cc.+2.5 mg. isocitrate/cc.	53
E-133	+ 5 mg. malonate/cc.+1 mg. malate/cc.	75
	+ 5 mg. malonate/cc.+1 mg. pyruvate/cc.	50

substantial increases of the respiration of tissue poisoned with 5 mg./cc. of malonate. That malonate inhibition of respiration could be in part overcome by the addition of succinate would be expected from the nature of the malonate inhibition of succinic dehydrogenase. This inhibition has been shown by Quastel and Wooldridge (35) and others to be competitive in nature and to depend on the close resemblance between the malonic and succinic acid molecules. Increase of the succinate concentration in the cell, with consequent decrease of the malonate/succinate ratio would, therefore, be expected to decrease the extent to which the enzyme is inhibited.

Malonate inhibition of spinach leaf respiration can also be overcome by the addition of fumarate to the system, as is shown in Table XV. Since reversal of malonate inhibition is a property specific to succinate, the fact that the inhibition is lifted *in vivo* by fumarate must signify either, a) that respiration can be carried on through oxidations involving fumarate, but not involving succinic dehydrogenase, or b)

that fumarate is oxidatively converted to succinate. That the latter is actually the case is strongly indicated by the experiments of Table XV. These show that, although the respiration of malonate poisoned leaves can be increased by the addition of fumarate, the respiration so increased can be inhibited by the addition of still more malonate. In other words, the respiration induced in malonate poisoned tissue by fumarate is a respiration in which succinic dehydrogenase is involved. It has not as yet been possible, however, to demonstrate directly by analysis that succinic acid is formed oxidatively from fumaric acid in malonate-poisoned leaves, owing to the fact that succinic acid, as well as malic and citric acids, is present in only low concentrations. Succinic acid was found to make up of the order of 0.01% of dry weight, and this in the presence of large amounts of other acids. The view that succinate is formed oxidatively in malonate-poisoned tissue is a conclusion similar to that drawn by Krebs and Johnson (29) from similar data on muscle. The data suggest that the oxidation of the  $C_4$  acids in the leaf may result in the reformation of the same substances, or in short, suggests that an organic acid cycle may be involved in respiration. That this cycle may be generally similar to the Krebs cycle is further suggested by the fact shown in Table XV that not only fumaric, but malic, isocitric and pyruvic acids are all capable of modifying to some extent the malonic acid inhibition of the respiration of leaf tissue. These acids should all be expected to be converted oxidatively to succinic acid through the operation of the cycle. Further evidence that the oxidation of pyruvate is linked with that of fumarate is contained in the experiment of Table XVI, in which respiration was

TABLE XVI

*Necessity of Pyruvic Acid for the Oxidation of Fumarate by Spinach Leaves  
in the Presence of Malonate and Fluoride  
(Expt. E-299)*

Treatment	O <sub>2</sub> uptake $\frac{mm.^3}{200 \text{ mg. leaf/hr.}}$ 2nd hour of treatment
Control	93
+5 mg. malonate/cc. +0.1 mg. NaF/cc.	17
+5 mg. malonate/cc. +pyruvate 2.5 mg./cc.	17
+5 mg. malonate/cc. +fumarate 2.5 mg./cc.	18
+5 mg. malonate cc. +pyruvate +fumarate	35



inhibited by fluoride and malonate simultaneously. The presence of fluoride will prevent the endogenous production of pyruvate, as shown in Table IX. Table XVI shows that, while neither added pyruvate nor added fumarate alone caused increased respiration in the fluoride- and malonate-inhibited tissue, the two together did bring about a significant increase in  $O_2$  uptake.

It has not been possible in the course of this work to demonstrate *in vitro* the presence of a succinic dehydrogenase or a succinic dehydrogenase system in any of numerous kinds of preparations made from spinach leaves, either by the Thunberg technique or aerobically in the respirometer. Berger and Avery (5) have reported a similar failure to find succinic dehydrogenase in *Avena* coleoptile preparations. That the failure to obtain an active succinic dehydrogenase *in vitro* in the present case may be due to extreme lability of the system is indicated by the following experiment.

Spinach leaves were chopped in the usual manner. One-half of the sample was then frozen with extreme rapidity on a block of dry ice and then thawed again as rapidly as possible. Samples representing equal amounts of fresh leaf were then placed in a series of Warburg vessels and oxygen consumption measured in the presence and absence of malonic acid. The frozen leaves were suspended in buffer of pH 6.7, approximately the pH of the whole cytoplasm, while it was necessary to suspend the intact leaves in buffer of pH 4.5 in order to insure penetration of the malonate.

Table XVII shows that, in the uninjured leaves, respiration was inhibited by malonate as usual. In the leaves which had been subjected

TABLE XVII

*Effect of Freezing and Thawing on the Total Respiration and on the Malonate-Inhibitable Portion of Respiration of Spinach Leaves*  
(E-124)

Treatment of lvs.	Inhibitor	pH	$O_2$ consumption in $\text{mm}^3/200 \text{ mg. leaf}/30 \text{ minutes}$			
			1st 10 min.	10-40 min.	40-70 min.	70-100 min.
Fresh leaves	None	4.5	50	54	47	38
Frozen leaves	None	6.7	42	27	14	14
Fresh leaves	Malonate 10 mg./cc.	4.5	28	25	14	12
Frozen leaves	Malonate 10 mg./cc.	6.7	23	22	13	14

to freezing the rate of oxygen consumption was greatly depressed as compared with the normal and the rate fell off rapidly with time. In addition, the effect of malonate became steadily less in the frozen leaves until, in the period 70–100 minutes, malonate exerted no influence at all on the respiratory rate. Since it would appear then, that succinic dehydrogenase largely disappears from leaf tissue within a short time after injury, it is not surprising that attempts to demonstrate the enzyme in cell-free preparations have thus far failed. It may be noted also that, in general, ground, lyophilized, or otherwise treated spinach leaf preparations always showed a very low rate of  $O_2$  uptake as compared to the normal intact leaf, as is shown in Table XVIII.

TABLE XVIII  
*Effect of Various Mechanical Treatments on the Respiration of Spinach Leaves*

Expt. No.	Treatment given leaves	mm. <sup>3</sup> $O_2$ consumed/ 200 mg. leaf/hr.
E-27	Whole leaves, scissor-chopped	167
	Ground in trituration mill	75
	Ground in colloid mill	5
E-36	Leaves lyophilized and scissor-chopped	12
	Leaves lyophilized, ground	6

## DISCUSSION

The course of respiration in the spinach leaf which is suggested by the above experiments is as follows: hexose is phosphorylated and converted through hexose diphosphate and phosphoglyceric acid to pyruvate. The phosphorylation may be brought about by enzymes present in spinach leaf *brei* and involves ATP. The pyruvic acid produced is oxidized through processes involving succinic dehydrogenase, and which may, in fact, involve the production from pyruvate of succinic acid itself. The evidence suggests the succinic acid may also be produced oxidatively from fumaric, malic or isocitric acid. These facts, as far as they go, are in full accord with the concept of the Krebs cycle. The oxidation of the individual plant acids, malic and isocitric, is mediated by dehydrogenases which require coenzymes present in yeast, possibly coenzymes I and II. The dehydrogenases may be readily studied *in vitro* and the protein apoenzymes have been prepared in a partially purified state. Succinic dehydrogenase, although it ap-

pears to exist in the intact leaf, is apparently labile and difficult to obtain *in vitro*. The succinic dehydrogenase of the spinach leaf differs from that of animal tissues in that it is not directly coupled to a cytochrome system. The terminal oxidase for all or essentially all of the oxidations of spinach leaf respiration appears to be polyphenol oxidase. The carrier or compound, which by reversible oxidation and reduction, couples the oxidase with other systems in the leaf has not been identified, although dihydroxyphenylalanine appears to be able to carry out this function. It has not been possible to build up coupled systems *in vitro*, using, for example, cytoplasmic protein, coenzyme I, malic acid and dihydroxyphenylalanine. Such a system, which contains both polyphenol oxidase and malic dehydrogenase, is capable only of oxygen absorption at the expense of the phenol substrate, and no coupling of phenol reduction with malic acid oxidation has been observed. This may mean that other as yet unrecognized enzyme systems and their carriers stand between the oxidase and the dehydrogenases in the living plant. It is, of course, obvious to think of flavo-proteins in this connection. Such flavo-proteins have not been detected in any large quantity among the proteins of the spinach leaf as yet, but they may, of course, exist in small amounts.

Cytochrome has not been found among the spinach leaf proteins and does not appear to play a role in spinach leaf respiration. The succinic dehydrogenase of the spinach leaf must differ, therefore, from that of animal tissues as well as from that of the wheat embryo, in that, with the previously known enzymes, transport of electrons has been confined to cytochrome as an acceptor. Whether, in the present case, succinic dehydrogenase once reduced is reoxidized by a quinone carrier or whether, here again, some other system intervenes, is not known.

#### SUMMARY

1. The respiration of spinach leaves has been investigated by *in vivo* studies of the gas exchange of excised leaf sections and by an examination of individual enzymes and enzyme systems *in vitro*.

2. Spinach leaves contain abundant polyphenol oxidase. Since both  $O_2$  uptake and  $CO_2$  evolution of spinach leaves are inhibited by inhibitors of this enzyme it is concluded that polyphenol oxidase is the principal terminal oxidase. The rate of spinach leaf respiration can also be increased by the application of dihydroxyphenylalanine, a substrate oxidizable by polyphenol oxidase.

3. *Brei* prepared from spinach leaves is capable of producing fructose diphosphate and glycerophosphate in the presence of glucose, and the amount of phosphorylated compounds produced is augmented in the presence of adenosine triphosphate.

4. The ability of spinach leaves to utilize glucose as a respiratory substrate is greatly reduced in the presence of fluoride. Pyruvate utilization is not, however, interfered with by fluoride, showing that in spinach leaves, as in yeast and muscle, fluoride may principally inhibit respiration by inhibiting the production of pyruvate from hexose. This fact, together with those given in 3 suggest that hexose breakdown in spinach leaves may follow a phosphorylytic pattern already well known for yeast and muscle.

5. Dehydrogenases for the oxidation of malic and isocitric acids were found in the leaf proteins of spinach leaves. These two acids as well as succinic, fumaric and pyruvic acids can be utilized as respiratory substrates by starved spinach leaves.

6. The respiration of spinach leaves is inhibited by malonic acid and it may be concluded that succinic dehydrogenase plays a part in the respiratory process. The inhibition caused by malonate can be reduced or abolished by the further addition to spinach leaves of succinate, fumarate, malate or isocitrate. These facts are in accordance with, but do not prove, the view that respiration in the spinach leaf is mediated by an organic acid cycle generally similar to the Krebs cycle.

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# Spectrophotometric Studies of the Oxidation of Fats VII. Oxygen Absorption and Chromophore Production in Lipoxidase-Oxidized Fatty Esters \*

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## INTRODUCTION

The oxidation of unsaturated fatty acids by the plant enzyme lipoxidase has been the subject of considerable study from the standpoint of oxygen uptake, peroxide formation and concurrent carotene destruction. A comprehensive review of the literature of this subject has been prepared by Sällman (1). It has been shown that, in addition to the effects mentioned, lipoxidase oxidation of certain substrates is also accompanied by a pronounced change in the absorption spectrum of the fatty substrate (2, 3). In an effort to correlate oxygen uptake with the spectral changes the present investigation was undertaken.

## EXPERIMENTAL

The methyl linoleate, ethyl linoleate, ethyl linolenate and methyl arachidonate used as substrates in these studies were prepared in the usual manner from their respective polybromides.

It was found that extraction of aqueous reaction emulsions with isoöctane, as used in the qualitative study reported earlier (2), gave rather poor recoveries of oxidized fat, although the isoöctane solutions were suitable for the demonstration of spectral changes accompanying oxidation. A means of extraction of the fat from the reaction emulsion which would give rise to a protein-free clear solution of fatty ester was sought. The procedure adopted was as follows.

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Approximately 0.25 ml. of fatty ester was weighed carefully and discharged under an inert atmosphere into a mixture of 5.0 ml. phosphate buffer, pH 7.0, and 5.0 ml. 1.0% dialyzed gum ghatti which was rapidly stirred with a stainless steel stirrer driven at high speed by an air turbine. The resulting emulsion was stable for an hour or longer. Equal portions of this emulsion (usually 0.50 ml.) were discharged by syringe into phosphate buffer (usually 2.0 ml.) in a series of conventional Warburg respirometer vessels, and equal volumes of dialyzed extract of defatted soybean meal (usually 0.50 ml.) were placed in the side cups. After the vessels had come to the temperature of the bath (13°C. unless otherwise stated) the systems were closed, and the reaction begun. At appropriate intervals of oxygen absorption individual vessels were removed from the bath, 2.0 ml. of the flask contents were removed and immediately discharged into 10.0 ml. methanol plus 0.5 ml. 1% zinc acetate solution. After chilling, the tubes were centrifuged and the clear supernatant fluid was used for spectrophotometric measurements. Blanks were prepared by treating the appropriate quantities of gum ghatti and enzyme solution with methanol and zinc acetate. When dilutions were necessary for measurements, methanol was used to dilute both sample and blank. To study the effect of alkali on the spectra, 2.0 ml. of the solutions of oxidized fat were treated with 2.0 ml. 20% aqueous alkali and diluted to 10.0 ml. with methanol immediately before spectrophotometric measurements were made. All spectrophotometric measurements were made using the Beckman spectrophotometer.

## RESULTS AND DISCUSSION

The enzymatic oxidation of the three esters proceeded so rapidly that one mole oxygen per mole ester was taken up in approximately five hours. This is in striking contrast to the rate of autoxidation which, at 37°C. with the same substrates, requires 20–100 hours to attain the same degree of oxidation, depending upon the substrate.

The development of the absorption maximum in the diene region (2300–2350 Å) in oxidizing methyl linoleate (Fig. 1) and in oxidizing ethyl linoleate (Fig. 2) was found to be a linear function of the oxygen uptake up to an oxygen content of 0.5 mole oxygen per mole ester ( $M/M$ ). The development of this band was accompanied by the appearance of another maximum at 2775 Å which did not show evidence of fine structure (Fig. 3). Neither of these bands in lipoxidase-oxidized linoleates was affected markedly by the addition of alkali. A comparison of these results with those obtained in a similar study of autoxidation of ethyl linoleate (4) will show the 2700 Å band induced during autoxidation is increased by the alkaline treatment. Aside from this discrepancy, the two oxidation processes seem to lead to similar results.

The results of three sets of experimental data obtained on the oxidation of ethyl linolenate at 13°C. are plotted together in Fig. 4. It will be seen that the development of the diene absorption band at 2340 Å

proceeds linearly with oxygen absorption up to about 0.4  $M/M$ . With greater oxygen content the absorption values become irregular and seem to show a tendency to decrease. Treatment of the oxidized ester with alkali has a negligible effect upon this spectral band in samples with less than 0.4 mole oxygen per mole. However, samples containing

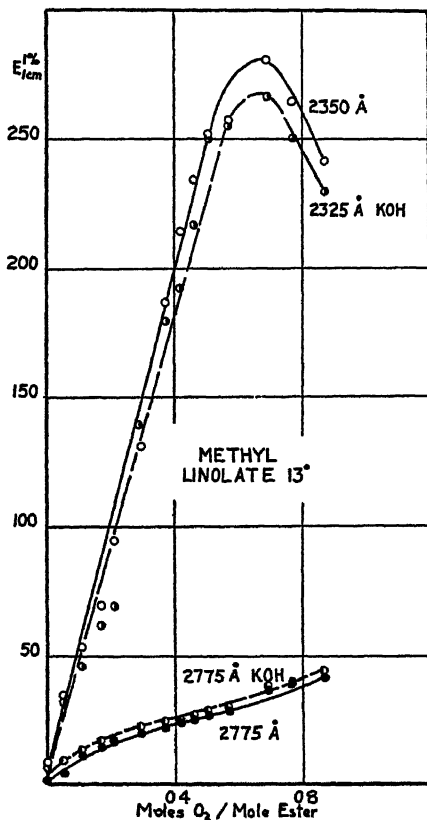


FIG. 1

Chromophore Production in Lipoxidase-oxidized Methyl Linoleate at 13°C.

more oxygen show a consistent and significant decrease in absorption at 2340 Å when treated with alkali. The reason for this abrupt change in the sensitivity of this chromophore to alkali is not understood. The enzymatic oxidation of linolenate also differs from that of linoleate in that its absorption bands at 2710 and 2800 Å rise significantly higher



than the 2775 Å band of oxidized linoleate, and in that these bands are extremely sensitive to alkali. Upon alkaline treatment the 2800 Å band increases significantly and an inflection indicating a new band near 3175 Å becomes apparent. This treatment is accompanied by the appearance of a yellow color. That the primary product of the lipoxidase oxidation of linolenates is less stable than that of linoleates is evidenced by the observations that with linolenates (1) the diene absorption maximum does not rise as high, (2) the linearity between

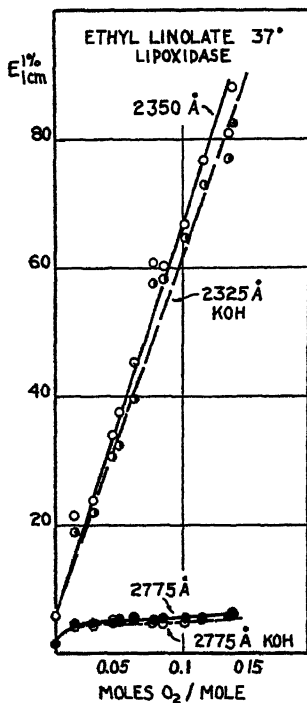


FIG. 2

Chromophore Production in Lipoxidase-oxidized Ethyl Linoleate at 37°C.

absorption in the diene region and oxygen content breaks down at a lower oxygen content, (3) the absorption in the longer wave lengths due to alkali-sensitive products is considerably greater and (4) the production of volatile products of oxidation is markedly greater as evidenced by an acrid odor.

A comparison of the chromophore-oxygen curves for lipoxidase oxidation and autoxidation (4) of linolenate again shows similarities and differences. The relationship between oxygen and the diene band shows approximately the same initial slope and irregularity toward the latter period of the oxidation in both cases. The bands in the 2700–2800 Å region seem to develop to a greater extent in the enzymatic oxidation, but treatment with alkali increases the absorption at 2800 and 3200 Å far more in the case of autoxidation.

The oxidation of methyl arachidonate by lipoxidase is accompanied by spectral changes similar to those found in the case of linolenate

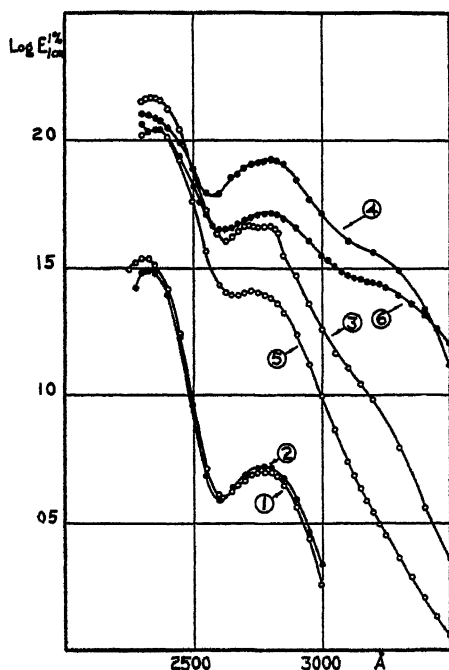


FIG. 3

Spectra of Enzymatically Oxidized Esters

- (1) Ethyl linoleate; 37°C.; 0.046 M/M
- (2) (1) in KOH
- (3) Ethyl linolenate; 13°C.; 0.818 M/M
- (4) (3) in KOH
- (5) Methyl arachidonate; 13°C.; 0.50 M/M
- (6) (5) in KOH

except that only a single band was found in the 2700–2800 Å region. The band appearing at 2350 Å is not markedly affected by alkaline treatment, but the band at 2725 Å increases markedly and shifts its maximum to 2800 Å. As in the case of oxidized linolenate an inflection appears near 3200 Å indicating the formation of another band (Fig. 3). This band was not measured in all samples and, hence, it is not plotted in Fig. 5 which shows the development of chromophores with oxygen

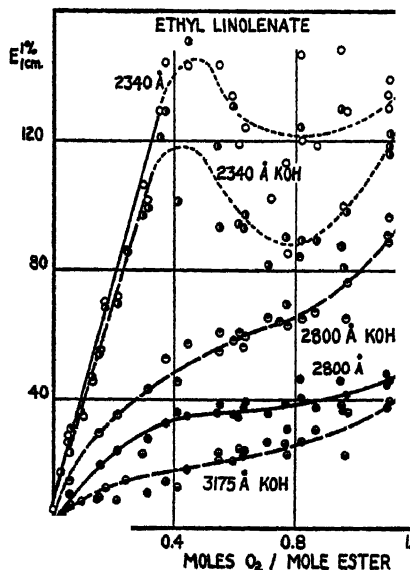


FIG. 4

Chromophore Production in Lipoxidase-oxidized Ethyl Linolenate at 13°C.

absorption. The relationship between oxygen content and extinction coefficient at 2350 Å seems to be linear although the values show some scatter. This linearity does not break down early in the reaction as is the case with linolenate, although arachidonate oxidation also leads to the formation of products sensitive to alkali.

A comparison of the chromophore-oxygen content curves for enzymatic oxidation of arachidonate and autoxidation of arachidonate (4) shows that the bands at 2350 Å develop at about the same rate with respect to oxygen in the auto- and lipoxidase oxidations. However, the enzymatic oxidation seems to favor the formation of the substance

absorbing at 2725 Å. On the other hand, autoxidation apparently leads to more of the substances sensitive to alkali.

In general it may be stated from the spectrophotometric study of auto- and lipoxidase oxidation that the two oxidations lead to similar products and that the enzymatic oxidation does not alter the yield of that product absorbing in the region of 2300–2350 Å, but that the

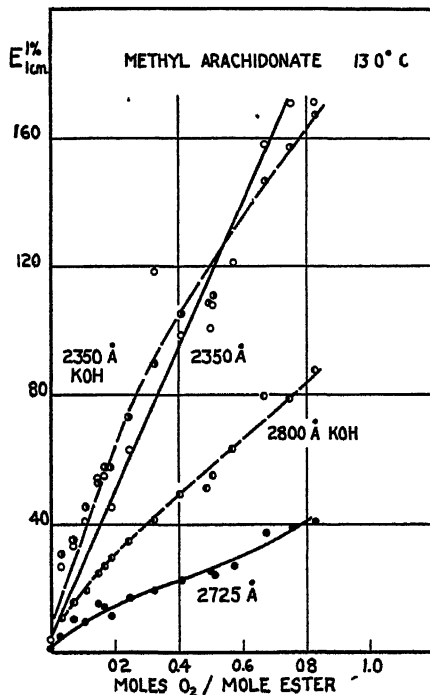


FIG. 5

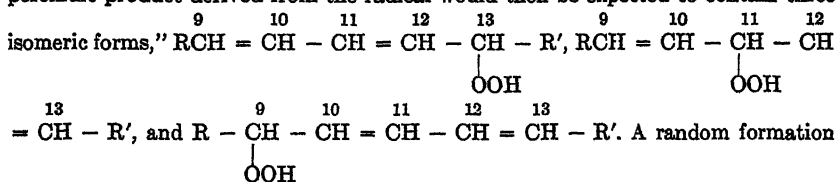
#### Chromophore Production by Enzymatic Oxidation of Methyl Arachidonate

yields of those products absorbing in the longer wave lengths are affected by the enzymatic oxidation. Whether the formation of secondary products of oxidation in the enzymatic oxidation is due to the enzyme lipoxidase or to some other fat oxidation enzyme present cannot be determined until this work can be repeated using a purified enzyme preparation such as reported by Balls, Axelrod and Keyes (5), and by Theorell, Bergström and Åkeson (6).

Recently Bolland and Koch (7) have shown that in the early stages of the autoxidation of ethyl linoleate all the oxygen absorbed can be accounted for as peroxides and that the molar extinction coefficient at 2315 Å is 22,700 for the primary oxidation product calculated on an oxygen basis. This they calculate to represent about 70% of the extinction coefficient of purified conjugated diene acids. From these observations the authors concluded that the primary thermal oxidation product is a diene-monohydroperoxide which is about two-thirds conjugated. In a study of the autoxidation of methyl linoleate Bergström (8) found essentially the same results but reported a molar extinction coefficient of about 14,000 for the primary oxidation product. By chromatographic separation Bergström was able to identify 9- and 13-hydroxystearic acids as products of the hydrogenation of the autoxidized methyl linoleate.

In a subsequent study of the oxidation of linoleic acid by lipoxidase Bergström (3) found that the oxygen absorbed could be accounted for as peroxides and that the absorption band at 2320 Å increased linearly with oxygen content. After hydrogenation of the reaction products he was able to isolate 9- and 13-hydroxystearic acids by chromatographic means. These results indicate that the reactions of lipoxidase oxidation and autoxidation are similar. Bergström concluded that the primary attack by oxygen is at the carbon 11 yielding a product which rearranges to yield two conjugated isomers, 9-hydroperoxido-10,12-octadecadienoic acid and 13-hydroperoxido-9,11-octadecadienoic acid.

Bolland and Koch (7) also postulate that the oxygen attacks the linoleate at the active methylene group between the double bonds to yield a "free radical which is a resonance hybrid composed of three equivalent canonical structures. The hydroperoxidic product derived from the radical would then be expected to contain three isomeric forms,"



of these isomers would result in a product which would be two-thirds conjugated, but it seems likely that the conjugated forms would be favored because of their resonance energy (7). Thus the isomerization induced by the oxidation of linoleate should result in a minimum of two-thirds conjugation.

The most probable values of the extinction coefficients of the monohydroperoxides of the linoleate, linolenate and arachidonate esters formed by autoxidation or enzymatic oxidation were calculated as follows.

The  $E_{1\text{cm}}^{1\%}$  value for the fresh ester was subtracted from the  $E_{1\text{cm}}^{1\%}$  values for oxidized samples of esters taken in the early stages of oxidation. These corrected  $E_{1\text{cm}}^{1\%}$  values and the oxygen contents expressed as moles oxygen/mole ester were used to calculate the slope of the straight lines representing the chromophore-oxygen content curves by the method of least squares,  $\Sigma \overline{E_{1\text{cm}}^{1\%}} \cdot \overline{\text{O}_2} / \Sigma \overline{\text{O}_2}^2 = \overline{E_{1\text{cm}}^{1\%}} / \overline{\text{O}_2}$ . The

value thus obtained for the  $E_{1cm}^{1\%}$  of product/mole oxygen was multiplied by one-tenth the molecular weight of the original ester to arrive at the molar extinction coefficient of the monohydroperoxide. This calculation is mathematically equivalent to that used by Bolland and Koch.

The choice of a primary standard value for the extinction coefficient of conjugated dienoic acids is difficult because the values reported in the literature vary greatly due to the use of different instruments and solvents (9). The value 25,000 for 10,12-linoleic acid in ethanol has been used in the calculations in this investigation rather than the much higher values obtained in nonpolar solvents, because all measurements in this study were made in methyl alcohol.

It will be seen from the table that there is a discrepancy between the extinction coefficients reported for methyl linoleate and ethyl linoleate. This is probably due to the inclusion in the calculations of samples with relatively high oxygen content in the case of methyl linoleate which would have the effect of reducing the slope of the  $E_{1cm}^{1\%}/O_2$  plot because of the greater formation of secondary products. In those experiments in which only samples from the very earliest stages of the oxidation of linoleate were used, the molar extinction approaches that reported by Bolland and Koch.

It seems unlikely that the diene conjugated systems induced by the oxidation of the three different fatty acids should have widely different

TABLE I  
*Extinction Coefficients of the Primary Oxidation Products  
of Unsaturated Fatty Acid Esters*

Ester	Type of oxidation	No. samples	Max. O <sub>2</sub> content	$\lambda_{max}$	$\epsilon$	Per cent conjugation
Methyl linoleate (Bergström (8))	37°C. Autoxidation	4	0.5M/M	2320 A	14,000	54.6
Methyl linoleate (Bergström (8))	37°C. (?) Lipoxidase	4	0.6	2320	13,000	50.7
Methyl linoleate (Bergström (8))	13°C. Lipoxidase	9	0.504	2350 2325 KOH	13,900 13,230	54.2
Ethyl linoleate (Bolland & Koch (7))	45°C. Autoxidation	5	0.19	2315	22,700	88.0
Ethyl linoleate	37°C. Autoxidation (4)	9	0.316	2325	18,960	74.0
Ethyl linoleate	37°C. Lipoxidase	12	0.14	2350 2325 KOH	19,360 17,470	75.6
Ethyl linolenate	37°C. Autoxidation (4)	5	0.379	2350	9,400	36.7
Ethyl linolenate	37°C. Lipoxidase	10	0.334	2390 2350 KOH	8,320 8,100	32.5
Ethyl linolenate	13°C. Lipoxidase	5	0.373	2340 2340 KOH	10,370	40.5
Ethyl linolenate	13°C. Lipoxidase	9	0.297	2340 2340 KOH	11,190 10,640	43.6
Methyl arachidonate	37°C. Autoxidation (4)	4	0.419	2350	6,740	26.3
Methyl arachidonate	13°C. Lipoxidase	9	0.411	2350 2350 KOH	8,860 8,240	32.6

extinction coefficients (7). It is more probable that the low absorption shown by the oxidized linolenate and arachidonate is due to the greater instability of the monohydroperoxides of these esters, polymerization of products of oxidation, or perhaps due to the partial formation of polyhydroperoxides. The observation that the bands produced in oxidized linoleates are not sensitive to alkali might indicate that the hydroperoxides are not destroyed by alkalinity or, at least, that the conjugated double bond system is unaffected. If such be the case, the alkali-sensitivity of the substances absorbing at the longer wave lengths in oxidized linolenates and arachidonates indicates that these products are not solely hydroperoxides. It is believed that the products absorbing at the longer wave lengths are largely decomposition products of the monohydroperoxides, probably unsaturated conjugated ketones, and that the lower absorption at 2350 Å in the oxidized linolenates and arachidonates is due to the greater tendency of the monohydroperoxides of these esters to undergo further reaction.

Before more than a qualitative significance can be attached to the apparent degree of conjugated diene produced during the oxidations of the fatty esters, it will be necessary to know the molar extinction coefficients of the pure monohydroperoxides; and before adequate comparisons can be made between this work and the observations of British and Swedish workers, it will be necessary to determine the extinction coefficients of a single sample of primary standard with the instruments used by these groups of workers.

In general it may be concluded that the autoxidation of the esters of linoleic, linolenic and arachidonic acids and the lipoxidase oxidation of the same lead to the same primary oxidation products. The subsequent reactions are qualitatively the same, but the conditions under which the oxidation takes place influence the amounts of the various secondary products.

### SUMMARY

1. A procedure whereby fatty acid esters and their oxidation products may be recovered from emulsion reaction mixtures for spectrophotometric measurement has been described.

2. The development of chromophores in lipoxidase-oxidized methyl linoleate, ethyl linoleate, ethyl linolenate and methyl arachidonate has been studied.

3. The development of the monohydroperoxide chromophore and the oxygen absorption of the system bear a linear relationship in the early stages of the oxidation.

4. The diene chromophore induced in the enzymatic oxidation of the esters is not appreciably affected by alkali. The 2775 Å band in oxidized linoleates is stable toward alkali, but the comparable bands in the oxidized linolenate and arachidonate are sensitive to alkali.

5. The calculated apparent molar extinction coefficients of the three ester monohydroperoxides arranged in decreasing order are linoleate, linolenate and arachidonate.

6. The molar extinction coefficients and the general shape of the chromophore-oxygen content curves for lipoxidase oxidation and for autoxidation of each of the esters are very similar, indicating that the two oxidation reactions are probably the same.

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# The Inhibiting Effect of Thiourea and Its Derivatives, and of Sulfur-Containing Amino Acids on the Formation of Iodocasein

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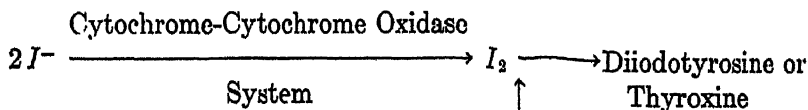
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## INTRODUCTION

In a previous communication, the remarkable capacity of thiourea and thiouracil to react with iodine has been demonstrated (1). Both substances are easily oxidized by iodine; in an aqueous medium, with formation of a disulfide, and in a phosphate buffer medium, with formation of higher oxidation products.

The results of those experiments suggested to us the possibility that such a marked reducing power toward iodine might play some role in the goitrogenic action of these compounds, in the sense that in the thyroid gland a competition for the iodine would take place between them or their oxidation products, and the aromatic amino acids which act as precursors of the thyroid hormone.

Thus, the path of the iodine leading from the iodides to diiodotyrosine or thyroxine would be blocked as shown in the following scheme:



To test this possibility a series of experiments has been carried out, in which iodine and casein have been allowed to react in the presence of goitrogenic substances,

Casein reacts with iodine to form iodocasein, which can be easily isolated and purified, and from which, after alkaline hydrolysis and fractional precipitation, substances can be isolated, which, from the chemical and physiological standpoint, are identical with diiodotyrosine

and thyroxine (2). This suggests the possibility that the mechanism which is responsible for the formation of thyroxine in the thyroid hormone is not intrinsically different from that which is involved in the formation of thyroxine in casein.

In the experiments to be reported below we have not confined ourselves to a study of thiourea and thiouracil but have also investigated the effects of derivatives of thiourea obtained by substitution at the nitrogen or at the sulfur, and of amino acids containing  $-SH$  or  $-S-$  groups.

#### EXPERIMENTAL

Since the thiourea and thiouracil were available only in the form of medicinal compounds, it was necessary to purify both substances. The former was recrystallized twice from ethanol, the latter (Malinckrodt) was dissolved in pyridine and precipitated in microcrystalline form by addition of an excess of water. The crystalline material was washed with alcohol and ether and dried to constant weight.

The phenylthiourea, cysteine hydrochloride, methionine and reduced glutathione were Eastman Kodak C. P. preparations. The S-methylthiourea sulfate (3) and the S-ethylthiourea hydrobromide were prepared in our laboratory (4). Since the last two substances are hygroscopic and decompose at room temperature, they were kept in a desiccator in the refrigerator.

Anal. Calcd. for S-methylisothiurea sulfate: N, 20.14; Found: N, 20.52.

Calcd. for S-ethylisothiurea hydrobromide: N, 15.48; Found: N, 15.14.

The casein was purified as follows: Casein (Merck, for nutritional studies) was dissolved in 1% NaOH. It was precipitated from this solution at pH 4.5 by addition of 10% acetic acid, the precipitate collected by centrifuging, and the isoelectric purification repeated. The purified material was washed in the centrifuge tubes first with acidified water (pH 5.0) and then with 95% ethanol. Finally, it was extracted with an excess of ether, the temperature of which was kept near the boiling point. The casein was first drained on filter paper and then dried by exposure to the air. A 1% solution of the dry casein in 5%  $NaHCO_3$  was used in our experiments. It was kept in the refrigerator when not in use.

The experiments were set up as follows: In each of a series of 150 cc. flasks 20 cc. of the casein solution (0.2 g. casein) was placed. To this the substance to be tested was added, in solution or as such, so that the amount added was in molar proportion to the iodine. In some cases the equivalent quantity of the compound with reference to the amount of iodine used was added, in others, one-half, one-fourth or one-eighth of this amount.

After the substance to be tested had gone into solution, water was added to give a volume of about 40 cc. From a burette a *N*/10 solution of iodine, always the same volume, was added to each flask, *i.e.*, 3.15 cc., corresponding to 0.04 g. iodine, as this quantity gave the best results in the iodination of the casein. The volume of the mixture was made up to 50 cc. with water. The flasks were kept for one-half hour in a water bath maintained at 37°C., after which they were cooled under the tap.

The iodocasein was isolated and purified as follows: From a burette, 10% acetic acid was added to each flask until a pH was reached at which the substance separated out (about pH 4.5, 16–17 cc. of acid being used). The casein was collected by centrifuging and dissolved in 10 cc. of 1% NaOH solution with stirring. With this solution the isoelectric precipitation was repeated, and the precipitate collected by centrifuging once more. The process of solution in alkali and of precipitation with acid was repeated. Finally, the iodocasein was washed twice with water and twice with 95% ethanol.

The remainder of the alcohol and moisture were removed *in vacuo*. The residue was spread on a glass dish and dried by exposure to a 75-watt electric bulb for 2 hours.

The iodocasein obtained was of a creamy color, the intensity of the color depending on the content of iodine. The same procedure was used to isolate and purify the casein from the blanks.

The iodine was determined according to the method proposed by Shahrock (5) for the estimation of iodine in dried thyroid. This worker claims to have used this method successfully on iodoprotein with an iodine content slightly above 13%. In spite of the fact that the method in our hands gave an error appreciably greater than that reported by Shahrock—differences of 0.6  $\gamma$  in duplicates of known samples—we believe that the accuracy is sufficient for the purposes of this investigation.

TABLE I

*Data on the Amounts of Iodine Combined with Casein in the Presence of Thiourea and Other S-Containing Substances*

Casein, 0.2 g.;  $I_2$ ,  $3.15 \times 10^{-4}$  g. equiv. Medium, 50 cc. 2%  $NaHCO_3$

Exp. No	Substance added	Amount added g mol. $\times 10^{-4}$	No of determinations	Per cent of iodine combined with casein	Per cent Standard Deviation	Per cent of iodine combined 100% = 7.54 $\pm 0.05$
1	none	—	16	7.84	$\pm 0.25$	100
2	Thiourea	3.15	5	0.08	$\pm 0.07$	0.38
3	Thiourea	1.57	2	0.56	$\pm 0.10$	6.55
4	Thiourea	0.785	1	2.30		28.90
5	Thiourea	0.392	1	3.26		41.21
6	Thiouracil	3.15	2	0.91	$\pm 0.32$	11.04
7	Thiouracil	1.57	2	3.59	$\pm 0.15$	44.45
8	Thiouracil	0.785	2	5.45	$\pm 0.30$	69.20
9	S-ethylthiourea					
	hydrobromide	3.15	1	7.69		98.7
10	S-ethylthiourea					
	hydrobromide	1.57	1	7.44		94.8
11	S-methylthiourea					
	sulfate	3.15	1	8.07		103.0
12	S-methylthiourea					
	sulfate	1.57	1	7.73		98.6
13	Methionine	3.15	3	1.06	$\pm 0.02$	12.97
14	Cysteine HCl	3.15	1	0.34		3.64
15	Glutathione	3.15	1	0.38		4.37
16	Phenylthiourea	3.15	2	0.44	$\pm 0.05$	5.00

17 Blank =  $0.05 \pm 0.043$  stand. dev. of 4 determinations

An inspection of the table shows that, with the exception of the S-substituted thiourea derivatives, the combination of iodine with casein is markedly decreased in the presence of the substances investigated. With regard to thiourea it may be said that when both this substance and iodine are used in equivalent quantities, the amount of iodine combined lies almost within the limits of the method used for the iodine determination. If the quantity of thiourea present is one-eighth of the molar equivalent of the iodine used, the quantity of iodine combined is 41% of the maximum amount taken up by casein in the absence of interfering substances.

The effect of thiouracil is perceptibly inferior to that of thiourea. This fact is not in harmony with the marked antithyroid action of this substance, but, as shown in our previous communication (1), the velocity of the reaction between thiouracil and iodine is slower, especially during the first half hour, while in the long run the reducing power of both substances is of the same order.

The introduction of alkyl groups into thiourea almost suppresses the inhibiting action of this substance, which is understandable since the reducing group has been blocked. Nevertheless, as shown in our experiments, some inhibiting action persists, 5% in the case of S-ethylisothiurea. We attribute this to the spontaneous decomposition which these substances undergo, especially at a higher temperature. We have found that the consumption of iodine by this compound increases gradually if the reaction is allowed to proceed at room temperature.

It is of interest in this connection that Astwood (6) has found methylisothiurea to be goitrogenic. We believe, however, that the discrepancy between his findings and ours is only apparent, as other workers (7) have reported this compound to act as a methyl donor. What seems probable is that such a labile substance decomposes in the body, giving rise to thiourea or to some other reducing compound.

The significance of the  $-SH$  group is shown plainly in the case of cysteine and glutathione; these compounds have a marked inhibiting effect on the combination of casein with iodine. It is a well-known fact that cysteine as well as glutathione are oxidized by iodine to form disulfides. The disulfide of the former, cystine, is oxidized by iodine in an alkaline medium to a greater extent (8).

Methionine occupies a peculiar position among the substances studied, its sulfur being present in a thioether linkage. Nevertheless, it has been demonstrated (9) that in an alkaline medium it is oxidized by iodine in two stages; first, two atoms of iodine combine with the S, then, as a result of hydrolysis, the iodine is converted into iodide and a sulfoxide,  $R-SO-R$ , is formed, whereby the velocities of both reactions depend on the pH of the medium and on the presence of iodine.

As was to be expected, substitution on the N does not alter the reducing power of thiourea. This substance has been reported to be goitrogenic.

Schachner *et al.* (10) have advanced conclusive proof to show that the system cytochrome-cytochrome oxidase plays a role in the iodination reactions taking place in the thyroid. In the introduction

we advanced a hypothesis to explain the antithyroid action of thiourea and its derivatives. According to this theory, the  $I_2$  formed by oxidation of ionic iodide under the influence of the cytochrome-cytochrome oxidase system cannot combine with the aromatic precursors of thyroxine because, to the extent to which it is formed, it reverts to its original reduced state under the action of these reducing substances.

Recently another example of inhibition of an oxidizing system by thiourea has been reported. Iodination of casein has been observed to take place in the presence of iodide under the influence of xanthine oxidase and of the peroxidase of non-pasteurized milk (11). This reaction is inhibited by thiourea.

### SUMMARY

It has been demonstrated that in a medium of 2% bicarbonate the combination of iodine with casein is inhibited by thiourea, thiouracil, phenylthiourea, and by amino acids such as cysteine, reduced glutathione and methionine.

Such an inhibiting effect is not shown by thiourea derivatives containing alkyl groups attached to the sulfur.

The bearing of the results obtained is discussed as a basis for the elucidation of the mechanism of goiter and of the inhibition of the thyroid hormone.

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# Carotenes of *Lycopersicon* Species and Strains<sup>1</sup>

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## INTRODUCTION

It has long been known that lycopene and  $\beta$ -carotene are normal constituents of red tomatoes. In addition, Went, Le Rosen and Zechmeister (1, 2, 3), in a series of three papers, have reported the presence of neolycopene A, neolycopene B,  $\gamma$ -carotene,  $\alpha$ -carotene, prolycopene and some unidentified carotenes in tomatoes.

In a research program for the development of tomatoes of higher provitamin A content (4) the present authors, in cooperation with members of the Department of Botany and Plant Pathology, have examined hundreds of tomato fruits produced from numerous crosses and selfs of the three species—*L. esculentum*, *L. hirsutum* and *L. pimpinellifolium*—for carotenes. The presence of unusual carotenes in fruits has been detected by spectroscopic examination of hexane extracts and demonstrated by chromatographic separation. Most selections studied had typical pigment systems (5). A few had either new carotenes or unusually high concentrations of pigments previously noted. The occurrence and identity of the major carotenes in *Lycopersicon* strains is reported in this paper.

## EXPERIMENTAL

The detailed analytical procedure for the more common carotenes in tomato selections has been described elsewhere (5). In brief, the following operations were performed. Representative fruits were homogenized in a Waring Blendor. A 20 g.

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sample was then extracted with an acetone-hexane mixture. After filtration the acetone layer was removed and the hexane fraction washed three times with distilled water. Carotenols and esters were removed from the carotenes by successive extractions with 90% methanol, 20% KOH in methanol and again with 90% methanol. The hexane solution was then washed with water, dried with sodium sulfate and made to volume. Absorption measurements were made with a photoelectric spectrophotometer (6).

For qualitative characterization, readings were made at wavelengths 4375 and 4875

Å. The value of the ratio  $\frac{\log \frac{I_0}{I}(4875 \text{ Å})}{\log \frac{I_0}{I}(4375 \text{ Å})}$  indicated whether a tomato selection con-

tained principally lycopene and  $\beta$ -carotene<sup>3</sup> or whether appreciable quantities of other carotenes were present.

When the ratio value was less than 0.85 or greater than 0.94, the hexane extract was chromatographed on a Ca(OH)<sub>2</sub> or MgO and Super Cel (one to one) column. Carotene bands were eluted with 10% ethanol in hexane after development of the column with 1-10% acetone in hexane. After removal of the polar solvents with water, the absorption curve of each pigment was determined. When hexane extracts with ratios indicative of the usual carotene system were chromatographed only traces of pigments other than  $\beta$ -carotene and lycopene were found.

To supplement the studies made on the carotenes of individual tomato selections, several bushels of fruit, the carotenes of which had odd light absorption ratios, were used. These were ground with an electric meat grinder and then dehydrated with ethyl alcohol. The remaining pulp was extracted several times with an acetone-hexane mixture. Acetone was removed by washing with water. The hexane phase was concentrated under reduced pressure and the carotenoids were chromatographed on a large MgO-Super Cel column. Several chromatograms were necessary to separate each of the carotenes.

## RESULTS

The relative positions, colors and names of the carotenes separated on MgO-Super Cel columns are listed in Table I. Various combinations of these carotenes were found in different *Lycopersicon* selections.

Typical characteristic absorption curves for each carotene band listed have been obtained from several sources. In Figs. 1, 2 and 3 characteristic curves for each carotene are shown. Those in Fig. 3 were plotted from original data; the others were taken from the literature. Zechmeister's data plotted in Figs. 1 and 2 agree well with data obtained in this laboratory.

Lycopene is a constituent of all red tomatoes. Its concentration varies greatly, however. In many yellow tomatoes and in green-fruited

<sup>3</sup>  $\beta$ -carotene and lycopene have nearly the same absorption ratio (i.e., 0.88 and 0.94, respectively).

TABLE I

*Position and Color, on a MgO-Super Cel Column, of the Principal Carotenes of Lycopersicon Fruit*

Color of zone	Substance
Red	all-trans-lycopene
Orange	neolycopene A
Clear space	
Red-orange	$\gamma$ -carotene
Clear space	
Yellow-orange	$\delta$ -carotene
Clear space	
Brownish-orange	polycopene
Clear space	
Yellow	unidentified I
Clear space	
Greenish-yellow	unidentified II
Clear space	
Pale green	all-trans- $\zeta$ -carotene
Clear space	
Orange	neo- $\beta$ -carotene U <sup>4</sup>
Red-orange	all-trans- $\beta$ -carotene
Yellow-orange	neo- $\beta$ -carotene B
Clear space	
Yellow	all-trans- $\alpha$ -carotene

species it is present in only trace quantities while, in some small-fruited *Lycopersicon pimpinellifolium* selections, as much as 300–400  $\gamma$  are present per g. of fresh fruit. Federal Plant Introduction selections 126953 and 127833 produce fruit with the greatest concentration of lycopene.

Neolycopene A concentration is principally a function of the lycopene content. Usually only 1  $\gamma$  of neolycopene A is found to every four or five of all-trans-lycopene.

$\gamma$ -Carotene has been observed in a large number of tomato selections (particularly red commercial types) but in none of these has it been found in greater concentration than about 8  $\gamma$ /g. of fruit.

$\delta$ -Carotene<sup>5</sup> is not a common constituent of commercial tomatoes or of other fruits and vegetables. It has been found to the extent of about 20  $\gamma$ /g. of fruit in several selections of the following pedigree: [Pritchard  $\times$  Pritchard  $\times$  F<sub>1</sub> (Pritchard  $\times$  *L. hirsutum* 127827)] ( $\otimes$ ).

<sup>4</sup> Observed only in canned samples.

<sup>5</sup> Strain (7) has reported the color and position of this carotene on magnesia and its light absorption maxima in hexane.

Polycopene has been found in a few selections to the extent of approximately 40  $\gamma$ /g. of fruit. The best sources of this pigment are the commercial varieties Tangerine and Golden Jubilee and the experimental selection Pan American  $\times$  F<sub>1</sub> [(Pan American  $\times$  *L. hirsutum* 126446)] ( $\otimes$ ).

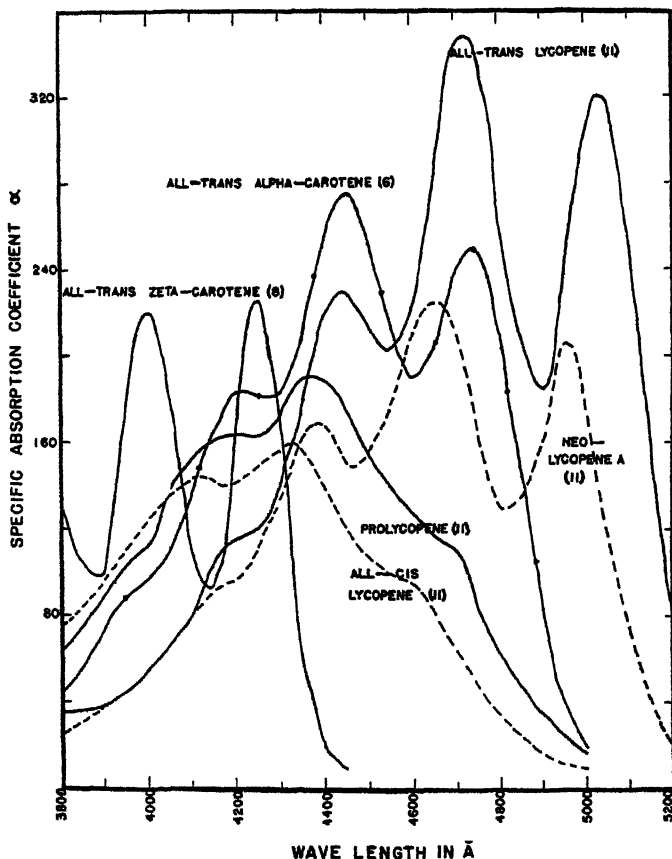


FIG. 1  
Specific Absorption Curves of Carotenes in Hexane

Unidentified carotene I has two maxima which agree in wave length with those of all-*cis*-lycopene (Fig. 1), but the lower wave length maxima and their characteristic absorption curves do not agree. The

absorption maxima are more distinct than those of all-*cis*-lycopene. The differences do not suggest isomerization of all-*cis*-lycopene or a mixture of all-*cis*-lycopene with carotenes normally adjacent on the adsorption column. In quantity this carotene seems to be related to polycopene. It is present in the same selections in slightly smaller quantities.

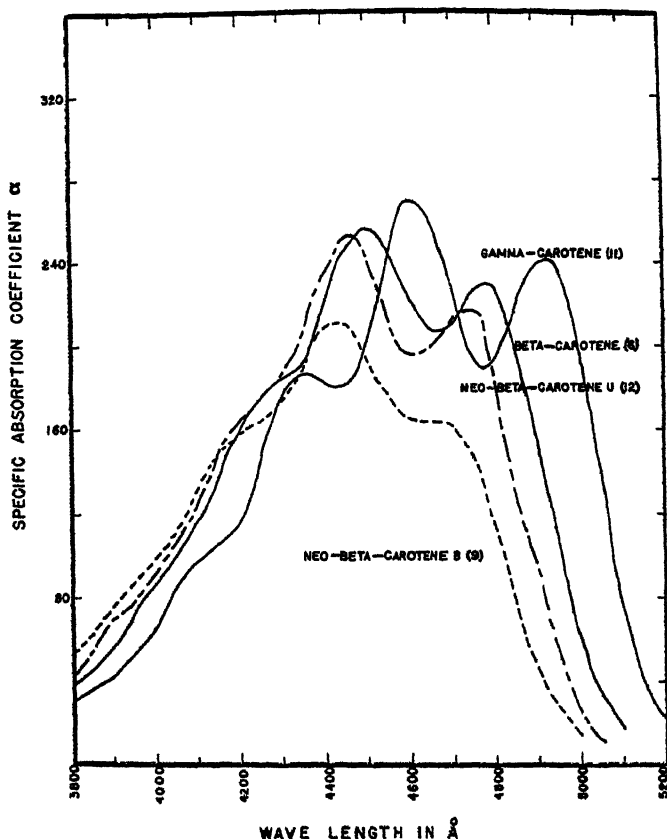


FIG. 2

Specific Absorption Curves of Carotenes in Hexane

Unidentified II has not been found in concentrations higher than 5  $\gamma$ /g. (estimated) in any tomato selections.

$\zeta$ -Carotene (8) is peculiar to several selections of tomatoes. Some of these had as much as 60-70  $\gamma$ /g of fresh fruit. The best selections have

the pedigree [Pan American  $\times$  F<sub>1</sub> (Pan American  $\times$  *L. hirsutum* 126446)] ( $\otimes$ ).

Neo- $\beta$ -carotene U has been observed only in canned tomatoes. Presumably it results from isomerization of  $\beta$ -carotene during the canning process.

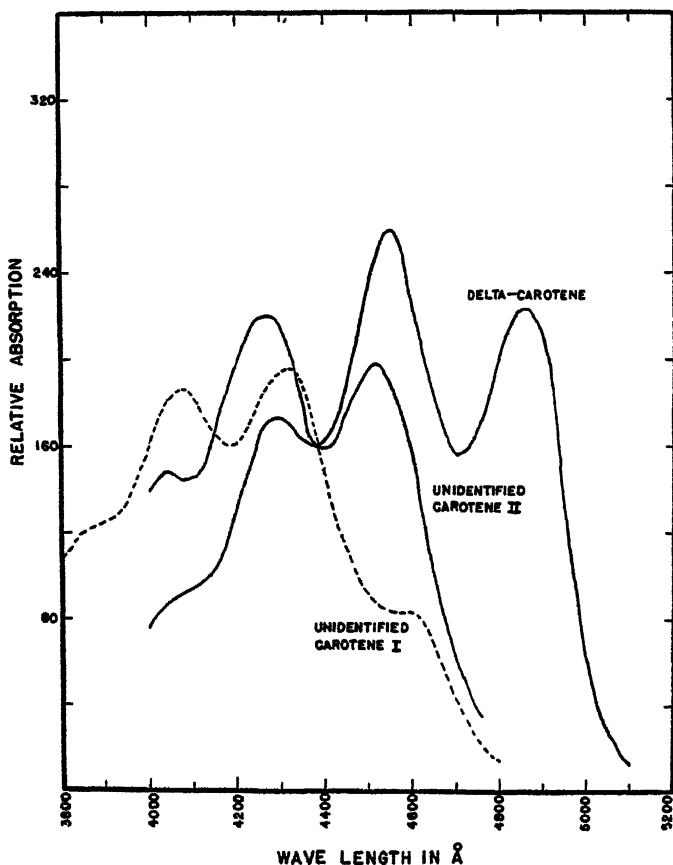


FIG. 3  
Absorption Curves of Carotenes in Hexane

$\beta$ -Carotene has been found in concentrations ranging from a trace to 130  $\gamma$ /g. of fruit. Such high concentrations are many fold the values found in present commercial varieties (2-8  $\gamma$ /g. of fruit) (4). Selections

of the following pedigree have synthesized the most  $\beta$ -carotene [(*L. hirsutum* 126445  $\times$  Rutgers)  $\times$  Indiana Baltimore] ( $\otimes$ ).

The content of neo- $\beta$ -carotene B is a function of the  $\beta$ -carotene content. About 20% of the  $\beta$ -carotene fraction obtained by chromatography is neo- $\beta$ -carotene B, comparable to values found in several other vegetables (9).

$\alpha$ -Carotene is not present in most tomatoes. In a few experimental selections of the pedigree [(*L. hirsutum* 126445  $\times$  Rutgers)  $\times$  Indiana Baltimore]  $\times$  (Marglobe  $\times$  F.P.I. 127827) as much as 15  $\gamma$ /g. of fruit have been observed.

### DISCUSSION

The development of selections within a species with a greater concentration of a known substance has been achieved previously through crossing and inbreeding. To the writers' knowledge, however, reported increases have not been nearly so great as that pertaining to  $\beta$ -carotene in *Lycopersicon*. The production of selections containing relatively high amounts of rare substances is new, but not entirely unexpected, when one considers the parental material used in this study. The green fruited parent *L. hirsutum* used in many of the crosses contained only small amounts of carotenoids. One may assume that the unusual carotenes found are either intermediates or side reaction products in the overall synthesis of carotenoids in tomatoes. Furthermore, one may suggest that new gene combinations produced by crossing and selfing are responsible for the production of these carotenes in appreciable quantities.

Little is known about the structure of  $\delta$ -carotene. From its absorption curve it may be assumed to contain the same number of double bonds as  $\gamma$ -carotene.  $\delta$ -Carotene was not found consistently associated with any one carotene in the various selections in which it was found.

Unidentified carotene II has nearly the same absorption maxima as the "T Pigment" described by Bauernfeind, Baumgarten and Boruff (10). They reported it to be formed from  $\beta$ -carotene on a column of alumina developed with chloroform. These workers were unable to separate "Pigment T" substance from "pigment C3a" (probably  $\zeta$ -carotene). In the writers' laboratory unidentified carotene II has been separated from  $\zeta$ -carotene on both alumina and MgO-Super Cel columns. The former gave good separation between the pigments when about 3% of ethyl ether in hexane was used, whereas the latter

column was developed with a small percentage of acetone (about 5%) in hexane.

The association of polycopene, unidentified carotene I, and  $\zeta$ -carotene in tomato selections was noted, and therefore it may be speculated that some relationship exists between  $\zeta$ -carotene and lycopene. The fact that their absorption curves are very similar in shape may be offered as further support for this speculation.

#### ACKNOWLEDGMENT

The authors wish to thank Dr. Ralph M. Caldwell, Dr. R. E. Lincoln, Mr. G. W. Kohler and Mr. Wayne Silver of the Department of Botany and Plant Pathology of Purdue University for making available from their tomato breeding nurseries materials used in this study.

#### SUMMARY

The principal carotenes found in fruit of *Lycopersicon* species have been separated by chromatography (MgO-Super Cel, 50-50) and characteristic absorption curves have been presented for each.

The relative position of the carotenes on MgO-Super Cel columns is as follows:

all- <i>trans</i> -lycopene	unidentified II
neolycopene A	all- <i>trans</i> - $\zeta$ -carotene
$\gamma$ -carotene	neo- $\beta$ -carotene U
$\delta$ -carotene	all- <i>trans</i> - $\beta$ -carotene
polycopene	neo- $\beta$ -carotene B
unidentified I	all- <i>trans</i> - $\alpha$ -carotene

Pedigrees of *Lycopersicon* selections containing the greatest quantity of most of these carotenes have been given.

These selections provide source material of the uncharacterized carotenes, breeding stocks for use in the production of commercial tomatoes of higher provitamin A content and genetic material for future inheritance studies.

The discrepancy between the absorption curves of all-*cis*-lycopene and unidentified I has been noted.

The association of  $\zeta$ -carotene and polycopene in several selections has been noted, and the possibility of a relationship between the former and lycopene has been mentioned.

The similarity of the absorption curves of unidentified II and the "T pigment" of Bauernfeind *et al.* has been mentioned.

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# Naturally Occurring Colorless Polyenes <sup>1</sup>

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## INTRODUCTION

In a research program for the development of tomatoes of higher provitamin A and vitamin C content (1) hundreds of *Lycopersicon* fruit have been analyzed for carotenoids. Some selections have been found to contain considerable quantities of new or little known carotenes while others contain large amounts of  $\beta$ -carotene (2). In many of the hexane extracts of *Lycopersicon* fruit (3) it was early noted that the solution fluoresced green when exposed to ultraviolet radiation. When these solutions were chromatographed on MgO<sup>3</sup>-Super Cel columns the fluorescent material (bluish-green) passed down the column ahead of the  $\alpha$ - and  $\beta$ -carotene bands. From the behavior of this substance it was assumed to be the same as the fluorescent material first detected by Strain (4) and recently described by Zechmeister and associates (5, 6, 7). Further examination of hexane extracts of *Lycopersicon* fruits spectrophotometrically (8) in the ultraviolet region established the fact that at least one more colorless polyene is present in *Lycopersicon* fruit.

## EXPERIMENTAL

Large collections of fruit containing appreciable quantities of the colorless polyenes were made.<sup>4</sup> The carotenes of these fruits were extracted, concentrated and separated

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<sup>3</sup> Adsorptive powdered magnesite No. 2641, Westvaco Chlorine Products, Newark, California.

<sup>4</sup> Selections of the following pedigree are good sources of the colorless polyenes. [Pan American  $\times$  F<sub>1</sub> (Pan American  $\times$  *L. hirsutum* 126446)]  $\otimes$ .

as described in a previous publication (2). The colorless polyenes were separated from the carotenes on a chromatographic column (MgO-Super Cel) with the aid of an ultraviolet lamp. A further separation was made between the fluorescent, colorless polyene and a non-fluorescent, colorless polyene by the same method. The latter precedes the former on a MgO-Super Cel column developed with 1-5% acetone in hexane.

*Properties of Colorless Polyenes*

On evaporation of the hexane under reduced pressure each of the colorless polyene fractions remains as a colorless to light yellow viscous oil which oxidizes readily. On

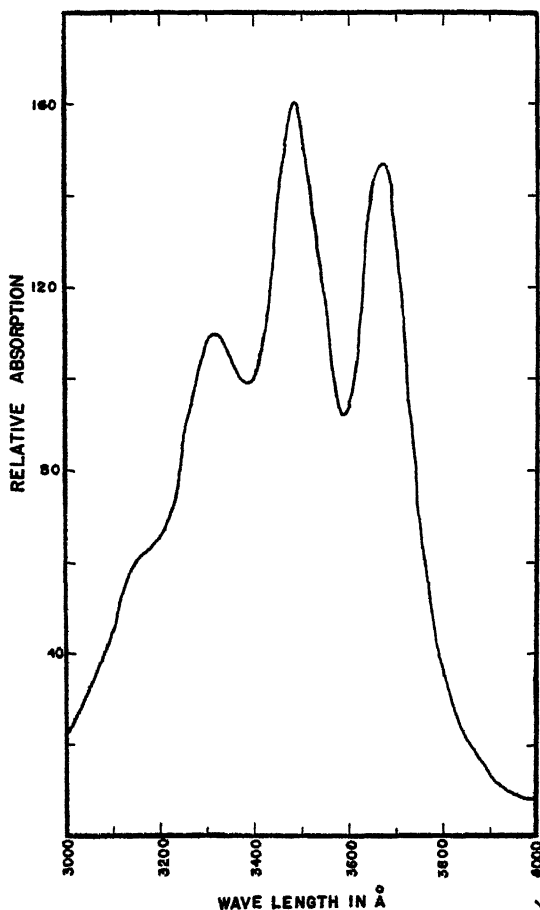


FIG. 1

Absorption Curve of Colorless, Fluorescent Polyene in Hexane

cooling to  $-20^{\circ}\text{C}$ . each solidifies. Attempts to crystallize each polyene at low temperatures by methods commonly used for carotenes have not been successful. When the first attempts at crystallization were made on the fraction containing the non-fluorescent, colorless polyene a large amount of crystalline material was obtained. This material however, had no absorption in the region 2200–4000 Å and, therefore, it was thought to be a saturated hydrocarbon or a mixture of several. Although the substance termed non-fluorescent, colorless polyene is non-fluorescent on a chromatographic column, it does fluoresce a pale blue color in a very concentrated solution of

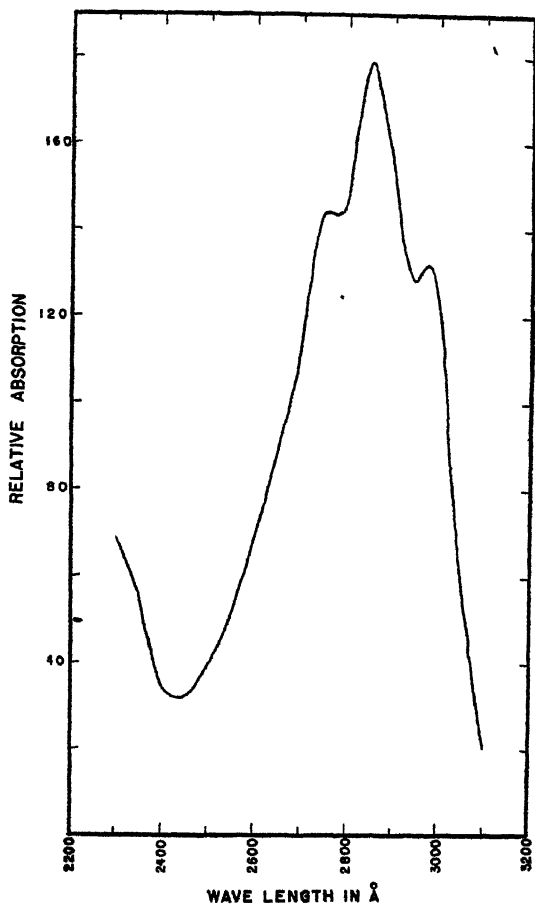


FIG. 2

Absorption Curve of Colorless, Non-fluorescent Polyene in Iso-octane

small volume. In partition studies, the polyenes were not transferred from hexane into 90% methanol before or after saponification. Each of the polyenes has a characteristic light absorption curve (Figs. 1 and 2).

### DISCUSSION

The colorless polyenes described in this paper are not the only ones occurring in tomatoes, for other fluorescent bands have been observed on MgO-Super Cel columns. The latter are present in very much smaller quantities, however, and may be largely isomers of the fluorescent, colorless polyene.

From the known properties of the two colorless polyenes (non-saponifiable, solubility, positive Carr-Price reaction, position on column and light absorption curves) it seems fairly certain that each is an unsaturated hydrocarbon. While the polyenes are present in many tomato selections, the importance of each is not known. It may be that each is related in some way to the more unsaturated carotenes (possibly intermediates in their synthesis). A definite structural similarity between the fluorescent, colorless polyene, anhydrovitamin A (9),  $\zeta$ -carotene and lycopene (2) is indicated by the similarity of the shape of their light absorption curves.

### ACKNOWLEDGMENT

The authors wish to thank Dr. R. M. Caldwell, Dr. R. E. Lincoln, Mr. Wayne Silver and Mr. George Kohler for making available from their breeding nurseries fruit of selections of *Lycopersicon* hybrids used in this study.

### SUMMARY

Two colorless polyenes have been obtained from fruit of *Lycopersicon* species. They have been separated by chromatography (MgO-Super Cel, 50-50).

The properties of the aforementioned substances indicate that each is an unsaturated hydrocarbon. The similarity of the shape of the absorption curves of the fluorescent, colorless polyene,  $\zeta$ -carotene, anhydrovitamin A and lycopene indicates a similarity in structure. The possibility that the colorless polyenes may be intermediates in the formation of carotenes has been mentioned.

The presence of other fluorescent, colorless polyenes in much smaller quantities in hexane extracts of *Lycopersicon* fruit has been observed. Also crystals of a saturated hydrocarbon or hydrocarbons have been obtained.

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## Succinic Acid Derivatives of Gramicidin and Methylol Gramicidin

A reaction product of gramicidin and formaldehyde has been found to have properties of possible therapeutic interest (1). The antibacterial activity of gramicidin is largely retained in this derivative, whereas the *in vitro* hemolytic action and toxicity are reduced. Chemically, the reaction results in the introduction of methylol groups, probably on the 2-position of the indole rings of the tryptophan residues (2). The presence of new hydroxyl groups in the derivative (hereafter called "methylol gramicidin"), corresponding in number to the indole nuclei known to be present, is readily demonstrated by means of quantitative acetylation (3) with acetic anhydride in pyridine.

It has now been found that esterification of the hydroxyl groups of methylol gramicidin with succinic anhydride yields a product, the sodium salt of which is completely water-soluble even in the presence of other ions.<sup>1</sup> This is in contrast to unmodified gramicidin which is so insoluble in aqueous media that its usefulness is greatly limited (4). The half-succinic acid ester derivative contains free acid groups corresponding in number to the hydroxyl groups known to be present in methylol gramicidin. It has approximately 25% of the antibacterial activity, 1-5% of the *in vitro* hemolytic activity and only 2% of the toxicity of gramicidin.

When gramicidin is treated directly with succinic anhydride, succinyl residues are introduced only on the hydroxyl groups.<sup>2</sup> The sodium salt of this derivative is antibacterially as active as gramicidin. It is soluble in distilled water containing 10% alcohol. It retains 20-50% of the original *in vitro* hemolytic activity, but apparently less than 2% of the *in vivo* toxicity. To reduce its hemolytic activity, it was sub-

<sup>1</sup> Part of the ester linkages are not stable at pH 6.85; about 20% were hydrolyzed in 20 hours at room temperature, and the material set to a gel (5-10% solution).

<sup>2</sup> The hydroxyl groups occur in the ethanolamine component. SYNGE, R. L. M., *Biochem. J.* 39, 355 (1945).



sequently treated with formaldehyde. Mild conditions were required, as the succinyl ester linkages were labile in the alkaline solutions used for the formaldehyde reaction. The most satisfactory preparation was slightly more water-soluble, as the sodium salt, than either gramicidin or methylol gramicidin, but less soluble than the neutral salts of the other succinyl derivatives described above.

TABLE I  
*Effect of Chemical Treatment on Properties of Gramicidin*

First treatment	Second treatment	Nitrogen	Acid groups	Solubility <sup>1</sup>	Anti-bacterial activity	Hemolytic activity <sup>4</sup>
		%	<i>Eqvts. per 10<sup>4</sup> gm.</i>	<i>mg./100 ml.</i>	%	%
None	None	14.5	0	0.6	100	100
Formaldehyde	None	13.8	0	2.5	80	20
Formaldehyde	Succinic anhydride	11.0	20	0.9 (>25 <sup>2</sup> )	25	1-5
Succinic anhydride	None	13.6	6.6	1.0 (3.3 <sup>3</sup> )	85	20-50
Succinic anhydride	Formaldehyde	13.4	2.2-4.1	2.3 (4.5)	75	10

<sup>1</sup> In 25 per cent alcohol solution containing 0.125 *M* sodium chloride. Figures in parentheses represent the solubility of the sodium salt.

<sup>2</sup> Completely soluble in water and dilute salt solutions.

<sup>3</sup> Was not precipitated from alcohol solution by the addition of distilled water.

<sup>4</sup> The hemolytic activity does not necessarily parallel toxicity (see text).

*Methods.* To 1 g. of gramicidin<sup>3</sup> or methylol gramicidin dissolved in 12.5 ml. of pyridine was added 1.5 g. of succinic anhydride. The mixtures were heated for 18 hours at 40-53°C. or for 1 hour at 80-95°C., then cooled, diluted with water and acidified with acetic acid. The insoluble derivatives were separated and washed by centrifugation, redissolved in alcohol and reprecipitated and washed with water, sodium chloride and/or acetic acid being used to flocculate the material. They were then frozen and dried. The sodium salts were prepared by suspension of the acid products in water and titration with sodium hydroxide to neutrality.

The procedure for formaldehyde treatment was as follows: To 1.4 g. of material, in 20 ml. of 95% ethanol were added 1 ml. of 1 *N* sodium hydroxide and 10 ml. of 40% formaldehyde. The mixture was held at 40°C. for 24 hours; then the product was isolated by dilution as described above.

The methods used for the assay of antibacterial (against *Staphylococcus aureus*) and hemolytic activities were those described previously (1).

Toxicity was estimated in a very limited number of rats by intravenous injection of solutions in propylene glycol, or as the sodium salts

<sup>3</sup> We are indebted to Wallerstein Laboratories for a generous supply of gramicidin.

in water. Gramicidin was found to be lethal in the dosage range 1.5–3 mg./kg., and methylol gramicidin, 9–15 mg./kg. (see also (5)). All succinic acid derivatives, when administered at levels up to 100 mg./kg., appeared to be noninjurious; the succinyl-gramicidin derivative after treatment with formaldehyde did not cause death in amounts up to 265 mg./kg.

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## Book Reviews

**Marine Microbiology.** By CLAUDE E. ZOBELL, Associate Professor of Marine Microbiology, Scripps Institution of Oceanography, University of California. The Chronica Botanica Co., Waltham, Mass., G. E. Stechert and Co., New York, N. Y. 1946. xv + 240 pp. Price \$5.00.

One of the long-standing "mysteries of the sea" is that of the marine bacteria and related organisms; their bacteriological and physiological characteristics, their numbers and distribution, and their rôle in the biological and physical economy of the world's largest environment. Lacking the ready accessibility and obvious practical applications that have, during the past several decades, spurred the enormous progress of general, medical, agricultural or industrial bacteriology, the microbiology of the sea has received, by comparison, so little study as to constitute practically a virgin field of research. Yet the problems are of profound theoretical interest as well as of much practical importance. There have been a number of observations and investigations, especially in recent years, but the literature as a whole has been widely scattered, frequently in more or less obscure publications. Thus, there is a genuine need for a comprehensive and critical review of the background, problems and present status of the subject. The book provides a notable contribution to this need.

The important data from diverse sources are gathered into an altogether readable, well organized discussion, illustrated by tables, diagrams and graphs. Following a brief historical background in the first chapter, the unique characteristics of the environment are given in adequate detail in the second; temperature, pressure, chemical composition, salinity, oxygen tension, pH, etc. In a full chapter the methods of collecting specimens and of enumerating or otherwise studying the microbial population of the water at various depths from the surface to the ocean bottoms, and the interpretation of the results obtained, are critically discussed before presenting the accumulated facts concerning the distribution, general and distinctive characteristics, and biochemical activities of the microorganisms. Successive chapters deal with the factors influencing distribution, the numbers and kinds of microorganisms in bottom deposits, their activities in bottom deposits, the peculiarities of marine bacteria, the aquatic yeasts and molds, the cycles of organic matter, of nitrogen, of sulfur and of phosphorus, the relation of marine bacteria, both parasitic and saprophytic, to the flora and fauna, and the microorganisms in marine air. In additional chapters due attention is given problems of purely practical and economic importance, such as the survival of human pathogens in sea water, the bacteriology of shell fish and other sources of food, the fouling of ship's bottoms or other submerged surfaces, and others. The last of the 18 chapters deals with the microbiology of inland waters, including both fresh and salt lakes, and the Dead Sea. The 22 pages of bibliography include some 600 references.

The available facts make the conclusion all but inescapable that the marine microorganisms are of prime importance in the cycle of life and transformation of matter in the sea, as in the soil. The evidence, however, is partly on the basis of quantitative studies, partly by inference, and much remains to be established.

Unfortunately, one fundamental aspect of the subject, viz., the influence of hydrostatic pressure on chemical rate processes and on biological reactions in particular, has, until recently, been so obscure that its full significance has remained largely unappreciated. It is hardly more than touched upon in the book. Yet nowhere in nature does hydrostatic pressure become so important a variable as in the sea, where the average pressure is nearly 5,000 pounds per square inch. The amount of metabolizable organic matter in the waters of the open sea is generally so low, and the bacterial population so sparse, that the significance of microbial processes there is uncertain. In contrast, the bottom deposits are rich in both, but they are generally under great pressures. It is now known that pressures of less than 5,000 pounds per square inch may profoundly influence the rate of enzyme reactions, rates of growth and disinfection of bacteria, denaturation of proteins, and other phenomena, and further, that the net effect of the pressure on biological processes is related in both direction and magnitude to temperature. Numerous temperature studies, referred to in the book, have been carried out under normal pressure in the laboratory, but they have no clear significance in the absence of additional pressure data. For, in organisms from the depths, pressure may be quantitatively more important than temperature, and the "pressure optimum" of growth and metabolism is quite as fundamental as the "temperature optimum." Perhaps the progress of investigations along these lines will provide data adequate for extensive treatment in subsequent editions of the book. It is no reflection against the first edition that they could not be included. The rational basis of pressure effects on reaction rates, and their relation to temperature, have been available, in the Theory of Absolute Reaction Rates, only since 1935, and have been applied in biology since 1942.

This book is a valuable and interesting introduction to, and summary of, the problems and status of marine microbiology. It is recommended not only to those who, unfamiliar with this phase of oceanography and microbiology, will want to widen their scientific horizon, but also to those who, actively engaged in the general field, will find it a convenient source of information and references.

FRANK H. JOHNSON, Princeton, N. J.

**Annual Review of Biochemistry, volume XIV.** JAMES MURRAY LUCK, Editor: Annual Reviews, Inc. Stanford University P. O. California, 1945, x, pp. 856. Price \$5.00.

Shortly after the advent of this volume, the reviewer bought it and spent many profitable hours perusing it in certain circumscribed fields of teaching and research. Later, when asked to review it, he spent many more hours reading it in detail and in checking many of its references. First may be noted possible shortcomings and deficiencies. The chapter on amino acids and proteins fails to cover the work of Sullivan and Hess, *J. Biol. Chem.* 155, 441 (1944), wherein the tryptophan content of several proteins was determined by four independent methods and an explanation was given as to the cause of wide variations reported in the literature. Also the chapter

fails in missing a valuable point in the field of denaturation. Thus Hess and Sullivan, *J. Biol. Chem.* **151**, 635 (1943), point out that the direct titration of the unhydrolyzed protein with iodine in the Okuda iodate method gives cysteine values of the same order of magnitude as determined in the acid hydrolyzate of the same protein—a finding that indicates that cysteine complexes are present in the native protein in a form comparable to certain thiazolidines. This finding changes the interpretation of the meaning of certain reactions associated with denaturation—the development of (SH) groups and so forth—and makes questionable the statement relative to reducing groups which are non-sulfhydryl, since some complexes of cysteine which will not give a nitroprusside reaction will react with iodine as though the (SH) group were free. In the chapter on sulfur compounds mention is made on page 266 of possible low methionine in legume seeds. Be this as it may, our experimental data indicate a fair amount of methionine in legume proteins. In the chapter on carbohydrate metabolism consideration is given to possible diabetes from alloxantin. The reviewer wisely says that the question requires further investigation. Our findings are that alloxantin in solution does not stay as alloxantin very long. In the chapter on nutrition attention is given to enriched bread, and on page 445 the reviewers are of the opinion that food habits (*i.e.*, liking for white flour) must be recognized. The present reviewer wonders if these habits should be recognized as potentially bad and needful of improvement. In the chapter on fat-soluble vitamins there is covered, page 546, the question of the beneficial effects of Vitamin E on muscular dystrophy. Without prejudice as to the value of Vitamin E in nutritional muscular dystrophy, we have found it of little remedial value in human muscular dystrophy. In this volume there is less reporting of experiments without statement of results than in earlier volumes. However, on page 350 such a condition obtains in the question of plasma lipids in patients with rheumatoid arthritis. Time would be saved for the reader if some comparative results were given.

On the whole the volume is a gold mine of information and reference worthy of much praise. The volume covers twenty eight topics of vital interest to workers in the field of biochemistry, 772 pages of current reading matter, 33 pages of authors' index, and 49 pages of subject index, and over 4500 references! Reading it thoroughly led to many new ideas and stimulated a number of research investigations in the reviewer's laboratory. Certain chapters seem exceedingly good. Among these may be mentioned The chemistry of the lipids; The chemistry of the amino acids and proteins; The chemistry and metabolism of the compounds of sulfur; The chemistry and metabolism of the compounds of phosphorus; Carbohydrate metabolism; Nutrition; Fat-soluble vitamins; The chemistry and metabolism of bacteria; and The chemistry of antibiotic substances, other than penicillin. To anyone interested in the progress of biochemistry the volume is a treasure house—well worth whatever time or money is spent upon it.

M. X. SULLIVAN, Washington, D. C.



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